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PRINCIPAL INVESTIGATOR: Xiaojiang Cui, Ph.D.
Adrian Lee

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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6. AUTHOR(S)
Xiaojiang Cui, Ph.D.
Adrian Lee

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: cui@bcm.tmc.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Both progesterone and the IGFs are critically involved in the development of the mammary gland and breast cancer. How they interact with each other to regulate breast cancer progression is not clear yet. In this study, we found that progesterone potentiates IGF-I action in breast cancer cells. Specifically, IRS-2 expression was markedly induced by progesterone, while IRS-1 and IGF-IR levels were not changed. This progesterone effect on IRS-2 was mediated by PR-B and via a transcriptiaonal mechanism. Furthermore, progesterone treatment enhanced activation of IGF-I-induced Erk and Akt signaling downstream of IRS-2. Interestingly, progesterone enhanced IGF-induced cell motility while showed no or little effect on cell cycle progression. This progesterone increase of IGF-I-induced cell motility was mediated by IRS-2 increase as blockade of IRS-2 expression by IRS-2 si-RNA transfection impaired the progesterone effect, which was probably not via Erk and Akt signaling. These data, together with previous findings that IRS-2 is activated by integrins, suggest that IRS-2 may be involved in progestin increase of IGF-induced cell motility. Our study may also provide a clue to the question of why progestins in hormone replacement therapy would enhance the risk of breast cancer in comparison to estrogen use alone.

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Introduction

Progesterone plays an important role in breast cancer development. Although progesterone is proliferative in the normal mammary gland, its inhibition of breast cancer cell growth in tissue culture has also been reported [1-3]. Studies from several groups have found that progestins exert a biphasic regulation of breast cancer cell growth — accelerating cells through the first mitotic cell cycle, then arresting them in G1 of the second cycle. Hence, it is proposed that progestins are inherently neither growth proliferative nor growth inhibitory, but rather sensitize breast cancer cells for growth factor and cytokine signals [4]. Human progesterone receptor normally exists in two isoforms, PR-A and PR-B, of 94 and 116 kDa [5, 6], with PR-B containing an additional 164 amino acids at its N terminus. The two PR isoforms display different transcriptional activities and are unequally expressed in different tissues and tumors [7-9].

The well-studied insulin-like growth factors (IGFs) also are key regulators of breast cancer development [10]. The IGF-I receptor (IGF-IR), upon activation by the IGFs, phosphorylates the insulin receptor substrates IRS-1 and IRS-2, which are multi-site adaptor proteins that link multiple downstream signaling pathways by binding to a variety of SH2 domain-containing proteins [11]. IRSs are also involved in signaling of insulin, interleukins, interferons, and growth hormone, and are implicated in breast cancer growth [12-14]. The IRS network of upstream and downstream signaling may place them in a central position to coordinate multiple signaling pathways. IRS-1 and IRS-2, despite their structural and functional similarities, are not completely interchangeable [15].

In the last few years, major effort has been focused on cross-talk between the IGFs and the estrogen signaling in breast cancer cells [16, 17]. However, how the IGFs cross-talk with progesterone in breast cancer is not clear. As a first step to raise the mysterious veil on IGF and progesterone interaction, I propose to study whether progesterone regulates focal adhesion and/or cell motility in breast cancer cells and whether this effect is via its regulation of IRS-1/2 expression and activation. The proposed training will prepare me technically for a career in the battle against breast cancer.

Body

1) Determine whether progesterone regulates focal adhesion in different breast cancer cell lines with distinct PR levels.

My previous studies failed to find progesterone regulation of focal adhesion in breast cancer cells that contained estrogen receptor (ER) and PR. To eliminate the potential confounding effect of ER, I stably transfected a specifically selected MCF-7 cell line C4-12 which is ER-/PR-[18], with PR-A or PR-B. Generation of these stable transfectants was described in the first annual report and these cells have now been reported in two of my publications in the journals Oncogene and Molecular Endocrinology. These cells have been sent to several other laboratories upon their request. These cells are very valuable in that PR can be studied without the masking effect of ER, as endogenous PR expression depends on estradiol. In addition, I can distinguish the effect of the two PR isoforms A and B.

I first tested whether progestins changed the cytoskeleton in C4-12/PR-A or C4-12/PR-B cells. It was found that progestins did not display significantly increased formation of stress

fibers in these cells. Additionally, they do not show any change in their ability to attach to matrices such as laminin or fibronectin. However, in collaboration with Doug Yee (University of Minnesota), he found using a modified Boyden chamber cell migration assay that C4-12/PR-B cells stimulated with progesterone exhibit markedly enhanced motility on fibronectin matrix in response to IGF-I (see Figure 1). Interestingly, progesterone does not potentiate IGF-I-elicited cell cycle entry.

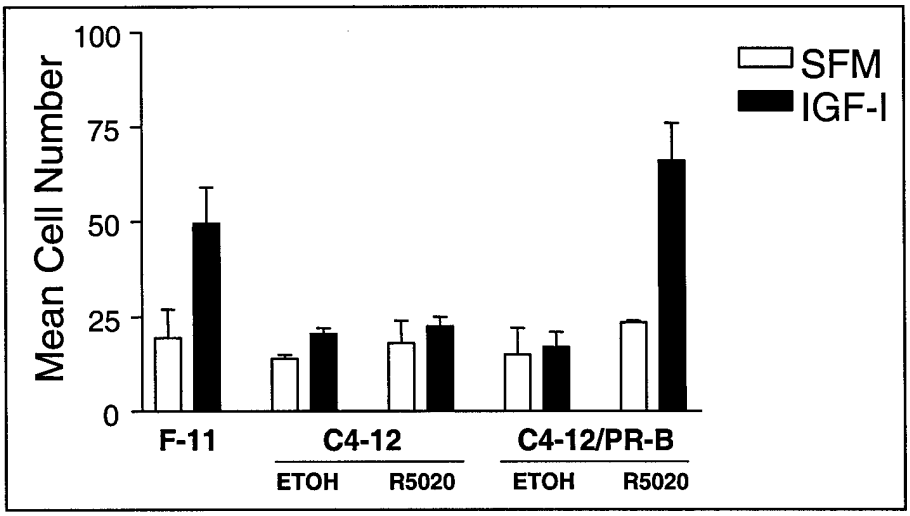

Figure 1: Progesterone sensitizes C4-12/PR-B cells to IGF-stimulated migration. Migration of C4-12, C4-12/PR-b and F-11 (variant of MDA-231 cells that migrate in response to IGF-I) was performed in a modified Boyden chamber assay with transwell coated with fibronectin. F-11 cells were used as a positive control. These cells were generated by successive passaging through mice via left ventricle heart puncture and generation of highly metastatic cells that have been previously shown to migrate in response to IGF-I and this is correlated with their increased IRS-2 levels compared to parental MDA-231 cells [19]. Cells were treated with wither vehicle (ETOH) or R5020 (10nM) and then treated with or without IGF-I (10nM). Cells that migrated across a transwell were counted and represented as mean cell number (y-axis). Bars represent triplicate wells +/- S.E.M.

I feel this is a very exciting discovery as it suggests that while progesterone may not regulate stress fiber formation similar to MDA-231 cells transfected with PR cDNA, it may still regulate IGF-dependent signaling events that lead to changes in migration. How progesterone regulation regulates IGF signaling is detailed in Aim 2. A manuscript about this work is under preparation.

2) Analyze whether progesterone regulation of focal adhesion correlates with changes of IRS-1/2 expression and activation

Surprisingly, I found that IRS-2 levels were dramatically upregulated by progesterone and the synthetic progestin R5020 in C4-12/PR-B cells, not in C4-12/PR-A cells, whereas IRS-1 and IGF-IR were not induced. Similar but weaker effects were observed in PR(+) MCF-7, T47D, and ZR-75 cells, possibly due to lower PR levels compared with C4-12/PR-B cells and potential inhibitory effect of PR-A on PR-B activity [20]. This progesterone up-regulation of IRS-2 is via transcriptional mechanisms. In addition, using immunoprecipitation with IRS-2 antibodies, I found that progestin treatment followed by IGF-I stimulation resulted in higher tyrosine-phosphorylated IRS-2 levels, increased binding of IRS-2 to Grb-2 and the PI3K regulatory subunit p85, and correspondingly enhanced ERK and Akt activation, as compared with IGF-I-only conditions. Since the similar results were obtained in C4-12/PR-B and other breast cancer cells with endogenous PR, it is reasonable to believe that the effect is a general characteristics of progesterone. This work has been published in the journal Oncogene. As IRSs are also involved in signaling of other growth factors, integrins, and growth hormone etc, progesterone may also increase the response of breast cancer cells to these signals. I think our data may have

implications in the investigation of why progestins significantly increase breast cancer risk in hormone replacement therapy.

3) Test the involvement of IRS-1/2 in focal adhesion induction by progesterone in breast cancer cells.

We have shown thus far that progesterone can increase IRS-2 levels, sensitize C4-12/PR-B to IGF signaling, and sensitized C4-12/PR-B cells to IGF stimulated migration. We are now studying whether the progesterone regulation of IGF-mediated migration is via the increase in IRS-2 levels. In collaboration with Doug Yee (University of Minnesota), he found specific si-RNA which can knock down IRS-2, not IRS-1, in breast cancer cells. When these siRNA was used to eliminate IRS-2 levels in C4-12/PR-B treated by progesterone, the progesterone effect on cell motility induced by IGF-I was impaired (Figure 2). In addition, the progesterone increase of IGF-I-induced IRS-2 signaling was also impeded. This result, together with two recent publications showing that increased IRS-2 enhances IGF-I mediated cell motility [19], and IRS-2 was found to be essential for the ability of integrins to promote cancer cell invasion [21], suggests that IRS-2 may indeed mediate the progesterone effect on IGF-I-induced cell motility. To investigate whether IRS-2 has similar roles in other breast cancer cells, I have generated IRS-1 and IRS-2 stably transfected ZR-75 cells. Preliminary results showed that IRS-2 overexpression can increase IGF-I-induced cell motility. Presently, kinase inhibitors are being used, e.g. U0126 (MEK inhibitor) and LY294002 (PI3K inhibitor) to examine which downstream proteins of IRS-2 is responsible for the progesterone regulation of cell motility in C4-12/PR-B cells. Preliminary data showed that Erk and Akt may not play a role in the progesterone effect. A manuscript about this work is under preparation to be submitted.

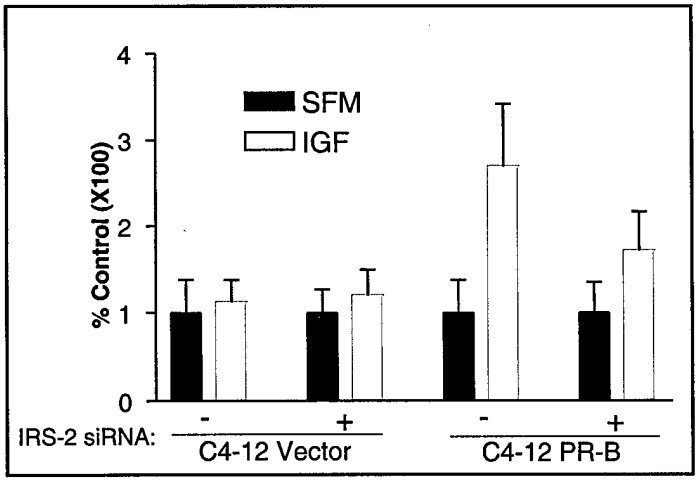

Figure 2: IRS-2 mediates the progesterone regulation of IGF-stimulated migration. Migration of C4-12 and C4-12/PR-B cells in response to IGF-I was performed in a modified Boyden chamber assay with transwell coated with fibronectin. Cells were transfected with IRS-2 si-RNA and treated with (10nM) R5020 and then treated with or without IGF-I (10nM). Cells that migrated across a transwell were counted and represented as mean cell number (y-axis). Bars represent triplicate wells +/- S.E.M.

While I was studying PR regulation of IRS-1 and –2, I also found that the IGFs regulate PR levels and activity in breast cancer cells. This work has now been published in Molecular Endocrinology. This is a very exciting observation that may provide a new paradigm for regulation of PR in breast cancer and may in part explain the unusual phenotype of ER+/PR- breast tumors that are unresponsive to antiestrogen treatment. This result may implicate that signaling inhibitors of small molecules may benefit treating ER+/PR- patients.

In addition to my work on PR regulation of IRS-2 and cell motility, I also wrote or co-authored three reviews in Clinical Cancer Research. I assisted in a project examining estrogen-

downregulation of E-cadherin which has been published in Cancer Research. We did not find significant effect from progestins on the E-cadherin expression. Given this fact and that estrogen and progesterone have distinct regulation of IRSs and IGF-IR, it is noted that these two important hormones affect breast cancer cell adhesion and motility in different mechanisms. This paper ties in with my work concerning the ability of steroid receptors to regulate cell adhesion, migration and invasion. Besides the above projects, I'm also working on several other projects in breast cancer. I expect several high quality manuscripts to be submitted in the near future. Here, I want to thank DOD for giving me the opportunity to receive valuable training in breast cancer research. Last year, I was promoted to Instructor position at Baylor College of Medicine. Because of the DOD fellowship, I am more than before motivated to pursue a scientific career in breast cancer research.

Key research accomplishments

- Generation of C4-12 cells stably transfected with PR-A or PR-B.
- Demonstration of progesterone induction of IRS-2 levels and increased sensitivity to IGF signaling in C4-12/PR-B cells.
- Demonstration of progesterone induction of IGF-induced cell motility in PR-B transfected C4-12 cells.
- Demonstration of IRS-2 essential in progesterone increase of IGF-induced cell motility.
- ZR-75 cells stably transfected with IRS-1 or IRS-2.
- Demonstration of IGF-I inhibition of PR levels and activity in breast cancer cells.

Reportable outcomes

- Cui X, Schiff R, Osborne CK, Lee AV (2004) Endocrine therapy of estrogen receptor positive progesterone receptor negative (ER+/PR-) breast cancer: New insights into the molecular mechanisms of resistance and clinical implications. J Clin Oncology (submitted).
- Cui X, Lee AV (2003) Regulatory Nodes That Integrate and Coordinate Signaling as Potential Targets for Breast Cancer Therapy. Clin Can Res. 10:396S-401S.
- Cui X, Lazard Z, Zhang P, Hopp, Lee AV (2003) Progesterone Cross-talks with Insulin-like Growth Factor Signaling in Breast Cancer Cells via Induction of Insulin Receptor Substrate-2. Oncogene. 22:6937-6941.
- Cui X, Zhang P, Deng W, Oesterreich S, Lu Y, Mills BG, Lee AV (2003) IGF-I Inhibits Progesterone Receptor Expression in Breast Cancer Cells via the PI3K/Akt/mTOR Pathway: Progesterone Receptor as a Potential Indicator of Growth Factor Activity in Breast Cancer. Mol Endocrinol. 17:575-588.
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- Lee AV, Cui X, Oesterreich S (2001) Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. Clin Cancer Res 7(Supplement) :4429-4435.
- C4-12 cells stably transfected with PR-A or PR-B.

- ZR-75 cells stably transfected with IRS-1 or IRS-2.
- Promotion onto Instructor position at Baylor College of Medicine.

Conclusions

I have stably transfected C4-12 cells with different isoforms of PR and found that PR-B allows cells to respond to progesterone by dramatically upregualting IRS-2 levels. This increase in IRS-2 is associated with increased response of the cells to IGFs. Correlating with this, progesterone treatment of these cells also allows the cells to migrate in response to IGF-I, which is mediated by IRS-2 signaling.

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Advances in Brief

Estrogen-mediated Down-Regulation of E-cadherin in Breast Cancer Cells[1]

Steffi Oesterreich,[2] Wanleng Deng, Shiming Jiang, Xiaojiang Cui, Margarita Ivanova, Rachel Schiff, Kaiyan Kang, Darryl L. Hadsell, Jürgen Behrens, and Adrian V. Lee

The Breast Center, Department of Medicine, and Department of Molecular and Cellular Biology [S. O., W. D., S. J., X. C., M. I., R. S., K. K., M. I., R. S., K. K., A. V. L.], Department of Pediatrics [D. L. H.], Baylor College of Medicine, Houston, Texas 77030, and Friedrich-Alexander-Universität Erlangen-Nürnberg, Nikolaus-Fiebiger-Zentrum für Molekulare Medizin, D-91054 Erlangen, Germany [J. B.]

Abstract

E-cadherin is an important mediator of cell-cell interactions, and has been shown to play a crucial role in breast tumor suppression. Its inactivation occurs through instability at its chromosomal locus and mutations, but also through epigenetic mechanisms such as promoter hypermethylation and transcriptional silencing. We show here that the potent mitogen estrogen causes down-regulation of E-cadherin levels in both normal and tumorigenic breast epithelial cells, and that this down-regulation is reversed by antiestrogens. The reduction in E-cadherin levels is via a decrease in promoter activity and subsequent mRNA levels. Chromatin immunoprecipitation assays revealed that estrogen receptor and corepressors were bound to the E-cadherin promoter, and that overexpression of corepressors such as scaffold attachment factor B resulted in enhanced repression of E-cadherin. We propose that estrogen-mediated down-regulation of E-cadherin is a novel way of reducing E-cadherin levels in estrogen receptor-positive breast cancer.

Introduction

E-cadherin is a glycoprotein with a large extracellular domain, a transmembrane domain, and a short intracellular domain that interacts with catenins. Recently there has been increased interest in E-cadherin as a mediator of cell-cell adhesions and as a tumor suppressor gene (reviewed in Ref. 1). E-cadherin maps to a region on chromosome 16q22.1 that shows frequent loss of heterozygosity in sporadic breast cancer. Although loss of heterozygosity-concurrent mutations have been found on the second allele in lobular breast tumors, very few mutations have been found in ductal breast carcinoma (1). This finding suggests that other epigenetic mechanisms such as hypermethylation and transcriptional silencing might play a role in E-cadherin inactivation. Indeed, methylation of the E-cadherin promoter has been shown to correlate with loss of E-cadherin expression in breast cancer cell lines and primary ductal and lobular breast cancers (2, 3). However, the decrease of E-cadherin expression is not simply attributable to hypermethylation, because treatment with 5-aza-2′-deoxycytidine fails to reactivate E-cadherin expression (4). Increased internalization and degradation via Hakai overexpression (5), as well as overexpression of transcriptional repressors known to inactivate the E-cadherin promoter such as Snail (6, 7) and SIP1/ZEB2 (8), are alternative mechanisms for its inactivation in breast tumors. Indeed, a recent study by Fujita *et al.* (9) showed that aberrant expression of Snail in ER[3] α (called ER throughout the manuscript)-negative breast cancer cell lines results in the loss of E-cadherin expression.

In this report, we show that, in ER-positive breast cancer cell lines, the steroid hormone E_2 down-regulates levels of E-cadherin protein and mRNA. This down-regulation can be reversed by antiestrogens used in the clinical management of breast cancer. We have evidence that the observed down-regulation depends not only on ER but also on the cross-talk with other pathways because it can be observed only when cells are kept in serum-containing media, not in serum-free media. The down-regulation involves direct recruitment of ER and ER corepressors at the most proximal E-cadherin promoter. This study is intriguing because (*a*) few estrogen-down-regulated genes have been described to date; (*b*) it provides evidence for a direct involvement of ER-corepressors (such as SAFB) in estrogen-mediated down-regulation of genes; and, finally (*c*) it presents a novel mechanism for E-cadherin inactivation in breast tumors.

Materials and Methods

Cells, Transfections, and CAT Assay. Human breast cancer cells (MCF-7L, MDA-MB-231, MDA-MB-435, T47D, ZR75) were maintained in IMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 200 IU/ml penicillin, 200 μg/ml streptomycin, and 6 ng/ml insulin. The immortalized human breast epithelial MCF10A cells were kept in DMEM/F12 medium supplemented with 5% horse serum, 10 ng/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 2 mM glutamine, 500 ng/ml hydrocortisone, 200 IU/ml penicillin, and 200 μg/ml streptomycin. To express ER in these cells, 5×10^5 cells were plated in a 6-cm dish and were transfected with 1 μg of HA-tagged ER (ER-HA-pcDNA3.1) for 5 h. To generate the ER-HA-pcDNA3.1 plasmid, we released the ER-HA cDNA from pcDNA3.1/V5/His-TOPO (10) with *Eco*RI, and ligated it into pcDNA3.1. After a 24-h recovery, the cells were placed in phenol red-free IMEM containing 5% CSS for an additional 24 h and then stimulated with 10^{-8} M E_2 for another 24 h before being lysed in 5% SDS for subsequent immunoblotting (see "Western and Northern Blot Analysis" below). The experiments were performed three independent times.

For reporter assays, cells were transiently transfected using Fugene (Roche, Indianapolis, IN) following the manufacturer's protocol. One day before transfection, cells were plated at 2×10^5 in 6-well plates. For E_2 induction experiments, the cells were treated either in SFM, which consisted of phenol red-free IMEM + 10 mM HEPES (pH 7.4) + 1 μg/ml fibronectin (Invitrogen, Carlsbad, CA) + trace elements (Biosources, Worcester, MA) + 1 μg/ml transferrin (Invitrogen) or in phenol red-free IMEM containing 5% CSS (Hyclone). Most E_2 induction experiments were performed at least twice; the experiments in MCF-7L cells were performed at least three times each. For the promoter analysis, 1 μg of E-cadherin promoter (−178/+92 bp) CAT construct (11) was transfected, and 24 h later, the medium was replaced with IMEM + 5% CSS containing the appropriate ligand. Forty-eight h later, cells were washed twice with PBS, and CAT activity was measured using the CAT

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[2] To whom requests for reprints should be addressed, at The Breast Center, Baylor College of Medicine, One Baylor Plaza, BCM 600, Houston, TX 77030. Phone: (713) 798-1623; Fax: (713) 798-1642; E-mail: steffio@breastcenter.tmc.edu.

[3] The abbreviations used are: ER, estrogen receptor (α); IMEM, Iscove's MEM; CSS, charcoal-stripped serum; SFM, serum-free medium; E_2, estradiol; IRS-1, insulin-receptor substrate 1; PgR, progesterone receptor; ChIP, chromatin immunoprecipitation; TAM, 4-hydroxytamoxifen; SAFB, scaffold attachment factor B; SAGE, serial analysis of gene expression; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ELISA from Roche (Indianapolis, IN). Values were corrected for protein concentrations and are presented as relative CAT activity. For transient transfections, triplicate samples were measured in each experiment. The data are presented as the average ± SE and are representative of three independent experiments.

SAFB1 Overexpression in MCF-7 Cells. To transiently overexpress SAFB1, subconfluent MCF-7L cells plated in a 10-cm dish were transfected overnight with 2 μg of SAFB1-HA-pcDNAI (12, 13) using Fugene (Roche, Indianapolis, IN) following the manufacturer's protocol. The next morning, the medium was changed, and 24 h later, the cells were lysed in 5% SDS. The generation of MCF-7 cells expressing inducible HA-SAFB1 has recently been described elsewhere (13).

Western and Northern Blot Analyses. Proteins were resolved on 8% SDS-PAGE, and electrophoretically transferred to nitrocellulose. The membrane was blocked in PBS/0.1% Tween 20 (PBST) + 5% milk for 1 h at room temperature. Antibodies to E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), IRS-1 (Upstate Biotechnology Inc., Waltham, MA), HA (Covance), SAFB (Upstate Biotechnology Inc.), PgR (Santa Cruz Biotechnology), ER (Vector, Novacastra, Burlingame, CA), and β-actin (Sigma, St. Louis, MO) were diluted at 1:1000, 1:1000, 1:1000, 1:500, and 1:5000, respectively, in PBST + 5% milk. After washing six times for 5 min each time with PBST, the membrane was incubated with horseradish peroxidase-linked antimouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) at 1:1000 in PBST + 5% milk and washed six times for 5 min each time, and the signal was developed using enhanced chemiluminescence according to the manufacturers instructions (Pierce, Rockford, IL).

For Northern blots, 10 μg of total RNA were separated by electrophoresis in a 1.2% formaldehyde-agarose gel. RNA isolation (CsCl gradient) and Northern blotting was performed after standard procedures. The human E-cadherin probe for hybridization was purchased from Research Genetics (Clone ID 2286727), and fold changes in RNA levels were determined using software on the Molecular Imager FX (Bio-Rad). The presented Northern blot is representative of two independent experiments.

ChIP assays. MCF-7 cells (3×10^6) were plated in 15-cm dishes in phenol red-free DMEM supplemented with 10% CSS. After 36 h, the cells were transfected with 250 ng of plasmid DNA (mouse E-cadherin promoter, pCAD-Ecad-3000; Refs. 11, 14) using LipofectAMINE and following the manufacturer's protocol. The next morning, the cells were treated with vehicle only, 10^{-8} M E_2, or 10^{-6} M TAM for 45 min. After washing the cells with PBS (three times), they were cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were rinsed three times with ice-cold PBS, were collected into 100 mM Tris-HCl (pH 9.4)-10 mM DTT, were incubated for 15 min at 30°C, and were centrifuged for 5 min at 2000 × *g*. Subsequently, cells were washed sequentially with 1 ml of ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)]. Cells were then resuspended in 0.3 ml of lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), protease inhibitors], sonicated three times for 10 s each time, followed by centrifugation for 10 min. Supernatants were diluted in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 250 μg were precleared with 2 μg of sheared salmon sperm DNA and protein G-Sepharose (40 μl of 50% slurry) for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with specific antibodies (2 μg protein/each). After immunoprecipitation, 50 μl of protein G-Sepharose and 2 μg of salmon sperm DNA were added, and the incubation was continued for 1 h. Precipitates were washed sequentially, each in TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], and buffer III [0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)]. Precipitates were then washed three times with TE buffer [10 mM Tris (pH 8)-1 mM EDTA], extracted with 1% SDS-0.1 M $NaHCO_3$, and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking. After DNA purification (QIAquick Spin kit), the proximal E-cadherin promoter (−234 to +62 bp) was amplified using the following primer set: forward primer, 5′-TCCTTTGTAACTCCATGTCTCCCGT-3′, and reverse primer, 5′-CGGGCAGGAGTCTAGCAGAAG-3′. The PCR of the pS2 promoter was performed as described previously (15). The antibodies for the ChIP assays were purchased from Santa Cruz Biotechnology (ER, N-CoR, rabbit IgG) and from UBI (SAFB). The experiments were performed three times.

Animals, Treatments, and Tissues. Animal care was in accordance with institutional guidelines. Female ovarectomized BALB/c athymic nude mice (4–6 weeks old; Harlan Sprague Dawley Inc., Madison, WI) supplemented with estrogen pellets (0.25 mg, Innovative Research, Rockville, MD) were inoculated s.c. with 5×10^6 MCF-7 cells, as described previously (16). When tumors reached a diameter of 7–9 mm (2–4 weeks), the animals were randomly allocated to continue estrogen treatment or to discontinue estrogen treatment by removal of the estrogen pellets. In this tumor model, estrogen stimulates tumor growth and estrogen withdrawal results in tumor growth inhibition. Tumors were removed during estrogen treatment (E_2 tumor group) and at 3 weeks after estrogen withdrawal treatment ($-E_2$ tumor group) and kept at −70°C for later analyses. Tumor powders were manually homogenized in a 5% SDS solution. After boiling and microcentrifugation, clear supernatants were collected, protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL), and Western blotting was performed as described above. This *in vivo* experiment was performed once.

Results

Estrogen Down-Regulates E-Cadherin Protein Levels in MCF-7 Cells *in Vitro* and *in Vivo*. To analyze whether E-cadherin protein levels are regulated by E_2, we placed ER-positive MCF-7L cells in a medium containing CSS and treated them with E_2 at a concentration ranging from 10^{-12} to 10^{-7} M for 24 h. Immunoblot analysis was performed using E-cadherin-specific antibodies, as well as β-actin antibodies for a loading control. As shown in Fig. 1*A*, E_2 treatment resulted in a dose-dependent decrease of E-cadherin. Interestingly, we never observed this E_2-mediated down-regulation of E-cadherin in the absence of serum, *i.e.* when the cells were kept in SFM (Fig. 1*B*). This result suggests that the E_2-mediated down-regulation of E-cadherin depends on other factors present in the serum, possibly "cross-talking" with ER.

Next we asked whether the down-regulation could be reversed by antiestrogens. Therefore, we treated MCF-7 cells with E_2 only, with the nonsteroidal antiestrogen TAM only, or with a combinations of both (Fig. 1*C*). As expected, the addition of antiestrogen to E_2-treated cells blocked E-cadherin down-regulation, reflecting the inactivation of ER activity. We observed the same effect with the pure steroidal antiestrogen ICI 182,780 (data not shown). As a control we immunoblotted for the estrogen-inducible proteins IRS-1 and PgR, levels of which were both potently increased by estrogen. Additionally, confirmation of ER function was shown by the down-regulation of ER (Fig. 1*C*) which is known to be degraded by E_2 and stabilized by TAM (17).

Interestingly, treatment with TAM alone increased levels of E-cadherin protein over baseline, reflecting the inhibitory effects of the residual E_2 in the CSS. Indeed, CSS can contain up to 10^{-11} M E_2 (data sheet from manufacturer). Further supporting this is our finding that increasing amounts of CSS resulted in a dose-dependent decrease of E-cadherin levels (Fig. 1*D*). We think that this is attributable to residual E_2 because (*a*) it can be reversed by TAM (Fig. 1*C* and data not shown); and (*b*) treatment with a range of growth factors such as epidermal growth factor, insulin-like growth factor-I (IGF-I), or heregulin in SFM did not lower E-cadherin levels (data not shown).

Next we asked whether this estrogen-mediated down-regulation of E-cadherin in MCF-7 cells in tissue culture could also be observed when MCF-7 cells were grown as xenografts in athymic mice. Therefore, athymic ovariectomized mice were given injections of MCF-7 cells, and tumors were allowed to grow in the presence of E_2 ($+E_2$). When the tumor reached 7–9 mm (2–4 weeks), the pellet was removed ($-E_2$), and the tumors stopped growing, as reported previously (16). We analyzed E-cadherin expression in estrogen-stimulated ($n = 4$) and estrogen-deprived tumors ($n = 4$). As shown in Fig. 1*E*, the E-cadherin levels were significantly lower in the $+E_2$ group as compared with the $-E_2$ group (Fig. 1*E*). Thus, estrogen treatment

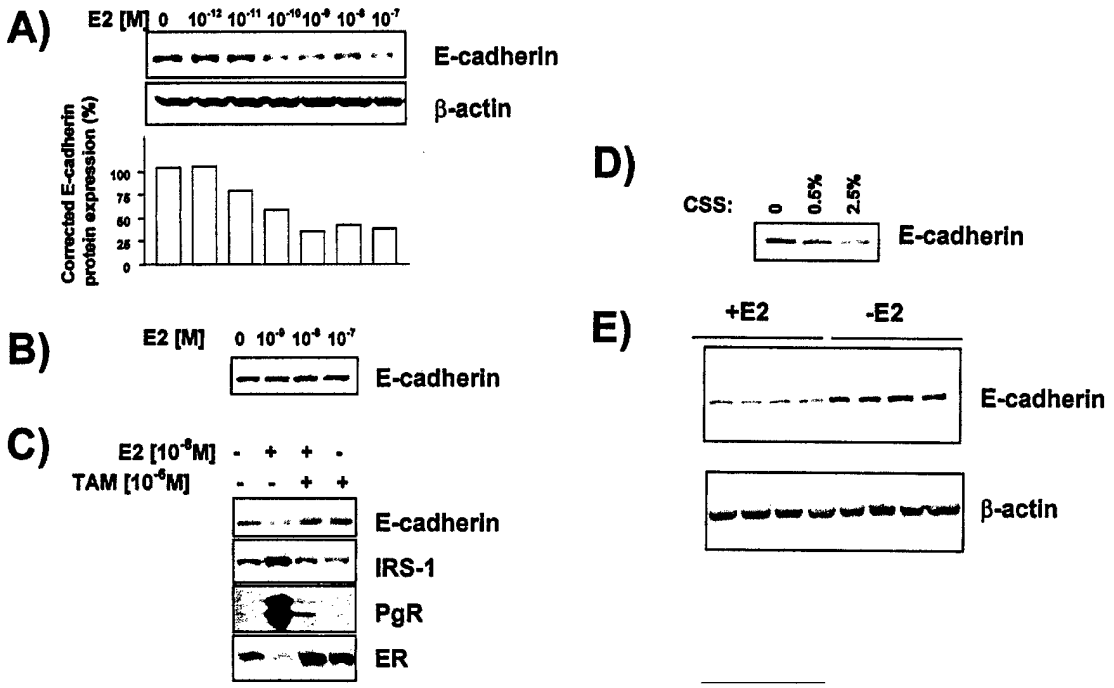

Fig. 1. Effect of E_2 and antiestrogen on E-cadherin protein levels in MCF-7 breast cancer cells grown in tissue culture and *in vivo* (xenograft). Proteins were extracted from MCF-7L cells and immunoblotting was performed using specific antibodies to E-cadherin (*A* through *E*), β-actin (*A* and *E*), IRS-1 (*C*), PgR (*C*), and ER (*C*). After enhanced chemiluminescence (ECL), images were captured using a CCD video camera (Fluorimager 8000; Alpha Innotech), and pixel intensity values were obtained with this machine. Values for E-cadherin were corrected for loading by dividing the E-cadherin pixel intensity by the β-actin pixel intensity. *A*, cells were grown for 48 h in 5% CSS and then were treated with increasing concentrations of E_2 for 24 h. *B*, cells were grown in SFM and then were treated with E_2 for 24 h. *C*, cells were grown for 48 h in 5% CSS and then were treated with E_2 and/or TAM for 24 h. *D*, cells were grown for 24 h in medium supplemented with increasing amounts of CSS. *E*, flash-frozen MCF-7 xenografts, grown in the presence and absence of E_2, were pulverized, and SDS extracts were analyzed by immunoblotting.

results in down-regulation of E-cadherin protein not only in MCF-7 cells grown in tissue culture but also *in vivo*. Although numerous (direct and indirect) factors can influence gene expression in an *in vivo* situation, we think that these data, together with our *in vitro* experiments, strongly support an estrogen-mediated down-regulation of E-cadherin.

Estrogen Down-Regulates E-cadherin Levels in Both Normal and Transformed Breast Epithelial Cell Lines. To exclude the possibility that the effect seen in MCF-7 was cell line-specific, we measured E-cadherin levels in two ER-negative breast cancer cell lines (MDA-MB231 and MDA-MB-435) and two other ER-positive breast cancer cell lines (T47D and ZR75). There was no expression in the ER-negative cell lines (data not shown), a finding that was recently described and analyzed by Fujita *et al.* (9). However, in the ER-positive cell lines, we detected estrogen-mediated down-regulation of E-cadherin, which was reversed by antiestrogen treatment (Fig. 2*A*).

We next asked whether this repression is specific to transformed cells, or whether it could also be found in cell lines with less severe genetic abnormalities. Because normal or immortalized breast epithelial cell lines do not express ER, we transiently transfected immortal but nontransformed MCF10A cells with ER to study E-cadherin regulation (Fig. 2*B*). Transient transfection of these cells with a green fluorescent protein-tagged ER construct revealed transfection of up to 10% of cells (data not shown). No ER was detected in cells transfected alone. Stimulation of ER-transfected cells with E_2 resulted in an increase in expression of the estrogen-regulated gene *IRS-1*, and a minor decrease in ER levels. This confirmed that the ER was active in these cells. Furthermore, these cells also showed a decrease in E-cadherin levels. In other experiments, the transient expression of ER alone (*i.e.*, not simulated with E_2) also caused a down-regulation, which is presumably caused by residual E_2 action; however, decreased E-cadherin levels were always noted after E_2 stimulation. Therefore, estrogen-medi-

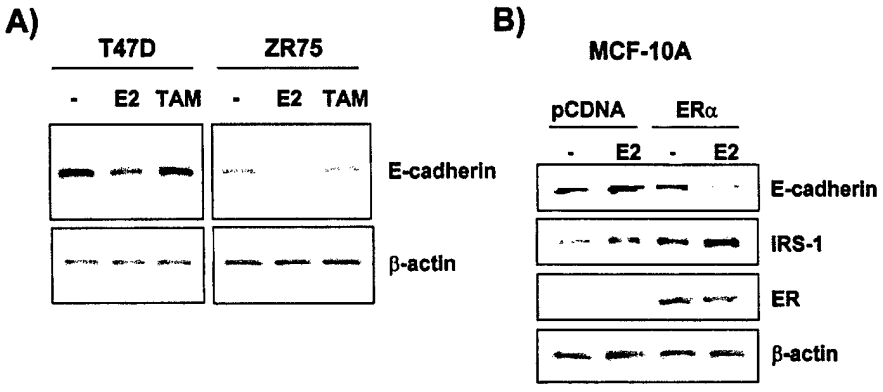

Fig. 2. Estrogen regulation of E-cadherin in immortalized and breast cancer cells. *A*, ER-positive breast cancer cell lines T47D and ZR75 were incubated in 5% CSS for 48 h, and, subsequently, were treated for 24 h with vehicle only, E_2 (10^{-8} M), or E_2 (10^{-8} M) and tamoxifen (10^{-6} M) in 5% CSS. β-actin was used as a loading control. *B*, immortalized MCF10A cells were transfected with ER-HA-pcDNAI and were treated with E_2 (10^{-8} M; $+E_2$) for 24 h. Cells transfected with empty vector only (pcDNA) served as negative control. SDS extracts were prepared and immunoblotted with antibodies as indicated.

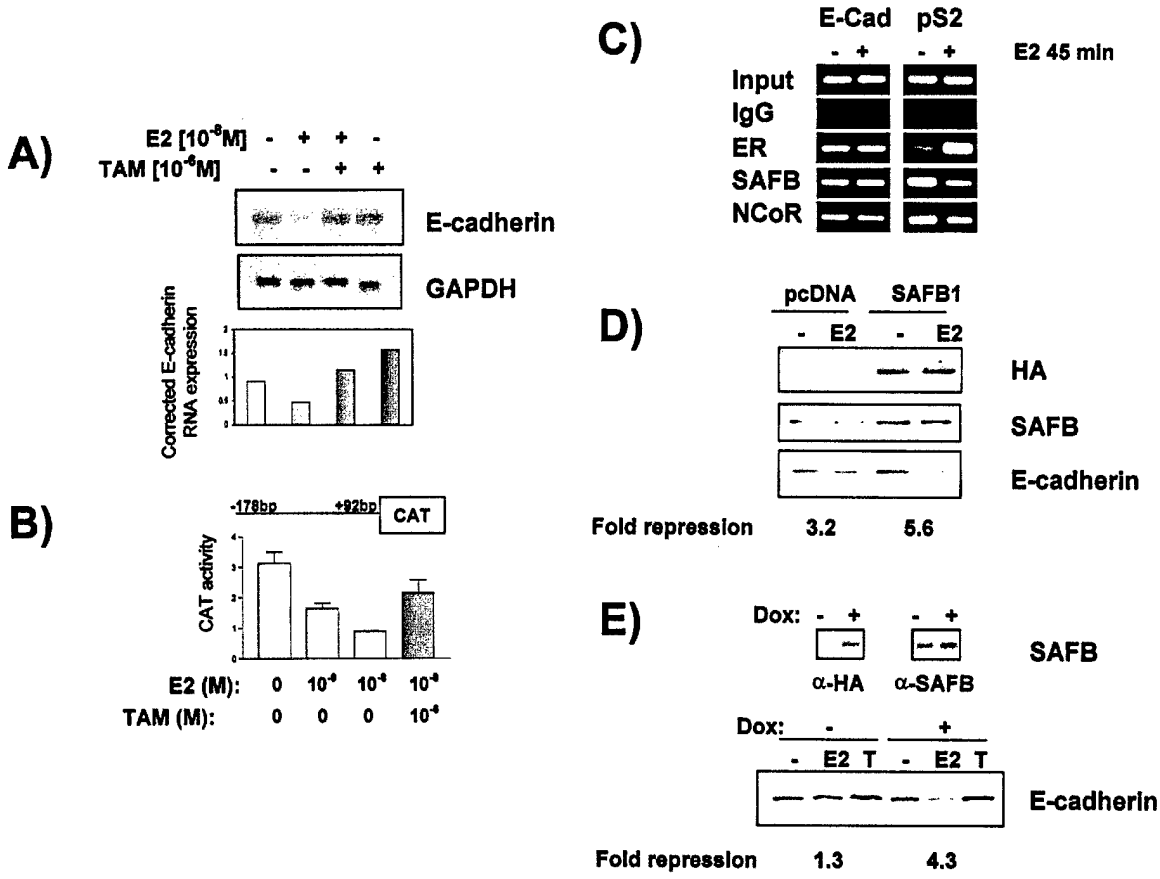

Fig. 3. Effect of E_2 on transcriptional regulation of E-cadherin. *A*, MCF-7L cells were treated with E_2 and tamoxifen for 10 h. After Northern blot analysis, data were quantified using a phosphorimager and were corrected for GAPDH. *B*, MCF-7L cells were transiently transfected with the E-cadherin promoter construct (−178/+92) and were treated for 24 h and CAT activity was measured. *C*, for the ChIP analysis, MCF-7 cells were transfected with the estrogen-responsive E-cadherin promoter. The next day, cells were treated for 45 min with vehicle only or with 10^{-8} M E_2, were cross-linked, and were subjected to immunoprecipitation and PCR as described in "Materials and Methods." *D*, MCF-7L cells were transiently transfected with vector only, or with SAFB1pcDNAI, and were treated with E_2 for 24 h. SDS extracts were immunoblotted, as indicated. E-cadherin levels were quantified as described above. MCF-7 cells that express tet-inducible SAFB1 (13) were treated with doxycycline for 48 h, and overexpression was confirmed by immunoblotting with HA and SAFB antibodies. Subsequently, cells were pretreated with doxycycline for 48 h, followed by treatment with vehicle only, 10^{-8} M E_2, or 10^{-8} M E_2 and 10^{-8} M tamoxifen. E-cadherin levels were quantified as described in Fig. legend 1.

ated repression of E-cadherin levels can be detected in both immortalized breast epithelial and cancer cell lines.

Estrogen Treatment Results in Decreased E-Cadherin RNA Levels and Promoter Activity. To determine whether estrogen decreased E-cadherin at the mRNA level, we treated MCF-7L cells with E_2 or a combination of E_2 and antiestrogen for 6 h and then isolated total RNA. Northern blot analysis was performed using an E-cadherin probe, with GAPDH as a loading control. As shown in Fig. 3*A*, E-cadherin RNA levels were decreased 2-fold in the presence of E_2. Substantiating the estrogen regulation at the RNA level is the finding that TAM treatment blocked the E_2-mediated down-regulation and, when given alone, caused an increase in the E-cadherin levels.

This finding encouraged us to analyze whether the E-cadherin promoter might be E_2-regulated in transient reporter assays. A number of studies have previously been conducted using a series of promoter constructs. We decided to use the most proximal E-cadherin mouse promoter construct (−178/+92 bp) which was previously shown to have strong activity in epithelial cells (11); however, it does not contain any classical estrogen response elements. This construct was transfected into MCF-7 cells, cells were treated with E_2 (10^{-9} M and 10^{-8} M), or with E_2 (10^{-8} M) and TAM (10^{-6} M) for 24 h, and CAT activity was measured. As shown in Fig. 3*B*, promoter activity was decreased in the presence of E_2, and, again, this repression was relieved by the addition of TAM. Thus, *E-cadherin* is an estrogen-down-regulated gene, and the down-regulation is mediated through the proximal promoter region.

ER Corepressor Proteins Are Recruited to the E-Cadherin Promoter, and Corepressor Overexpression Results in Enhanced E-Cadherin Repression. To analyze whether the decreased promoter activity was the direct result of recruitment of ER and corepressors, we performed ChIP assays. Therefore, we transfected MCF-7 cells with a plasmid containing the E-cadherin fragment known to be repressed on estrogen treatment. The cells were then treated with E_2 for 45 min, and the recruitment of ER and corepressors (N-CoR and SAFB1) was analyzed by ChIP (Fig. 3*C*) as described in "Materials and Methods." We also examined the recruitment of ER and corepressors at the pS2 promoter. As described previously (15), estrogen treatment resulted in strong recruitment of ER to the pS2 promoter. We repeatedly detected a low level of ER binding in the absence of E_2, possibly reflecting low levels of E_2 in the stripped serum. Corepressors (N-CoR and SAFB1) showed constitutive binding to the pS2 promoter, which was released on E_2 treatment. In stark contrast to results obtained with the pS2 promoter, the E-cadherin promoter showed strong constitutive binding of ER in the absence of estrogen. E_2 treatment of cells with estrogen for 45 min did not result in release of corepressors, as observed for the pS2 promoter (Fig. 3*B*).

To directly test whether the corepressors might modulate E-cadherin levels, we transiently overexpressed the ER corepressor

SAFB1, and also generated stable tetracycline-inducible SAFB1 overexpressing breast cancer cell lines (Fig. 3*D*). Transient overexpression of SAFB1 in MCF-7L cells resulted in stronger estrogen-mediated repression of E-cadherin, as shown in Fig. 3*D*. We confirmed this data in MCF-7 RTA ("tet on") cells which consistently showed 3-fold overexpression of SAFB1 on doxycycline treatment (Fig. 3*E*). Thus, ER corepressor levels are a major determinants in the regulation of *E-cadherin* expression.

Discussion

In this study, we have shown that *E-cadherin* is an estrogen-down-regulated gene in human breast cancer cells. A number of studies in various tissues and cell lines have previously described connections between steroid receptor pathways and E-cadherin. Prinsac *et al.* (18) and Habermann *et al.* (19) have shown that developmental exposure to estrogen was associated with changes in epithelial cell adhesion and decreased E-cadherin levels in the adult rat prostate. E_2 treatment resulted in a decrease of N-cadherin (20) and increase of E-cadherin (21) levels in the mouse ovary. In breast cancer cells, estrogen treatment was reported to induce cytoskeletal rearrangements (22) including delocalization of E-cadherin (23). Interestingly, tamoxifen restored the function of E-cadherin in an MCF-7 subline with a functionally inactive cell surface E-cadherin (MCF-7/6); however, this was an extremely rapid event (30 min) and did not require protein synthesis (24). Thus, although a number of reports have addressed a potential effect of estrogen on E-cadherin, our study represents the first attempt to investigate estrogen-mediated down-regulation of E-cadherin as a novel mechanism of its inactivation in human breast cancer.

In contrast to the well-characterized estrogen induction of a number of genes, estrogen-mediated down-regulation of genes has only recently gained more attention. In a SAGE study using estrogen-treated MCF-7 cells, an equal number of induced and repressed genes were identified (25). A recent study has shown that transcription of the nuclear coactivator src-3/AIB1 (amplified in breast cancer) is repressed by E_2 (26). We think that estrogen-mediated repression of genes is a critical regulatory pathway in ER-positive cells, and that deregulation of this repression in breast cancer may have dramatic effects such as the promotion of transformation and metastasis. The observation that a number of genes, including *E-cadherin*, have been described as both induced and repressed might be explained by our finding that the repression can only be seen in the presence of serum but not in SFM, suggesting that cross-talk with other pathways is necessary. For instance, kinases can regulate ER and coregulators (for a recent review, see Ref. 27), and the absence or presence of a specific kinase might determine whether the gene becomes induced or repressed on estrogen treatment. We hypothesize that there are three distinct sets of genes: one that can only be induced by estrogen, one that can only be repressed by estrogen, and one that can be induced or repressed depending on cellular context. Experiments are ongoing to test this hypothesis.

As for the mechanism of estrogen down-regulation of gene expression, our ChIP analyses suggest an involvement of ER corepressors and ER in a complex at the E-cadherin promoter. Several previous studies have suggested that a balance of coactivators and corepressors may modulate ER action, and may be deregulated in breast cancer and, in particular, in endocrine resistance. Our data support this hypothesis by showing that an excess of SAFB1 enhances the ability of ER to down-regulate E-cadherin levels. Thus, our data imply that a critical balance between ER and ER cofactors is a determinant in the regulation of E-cadherin levels in breast cancer. However, a potential caveat of our experiments is the use of a transiently transfected promoter. Studies analyzing the recruitment of both coactivators and corepressors to the endogenous E-cadherin promoter in mouse and human cell lines are ongoing.

The connection between ER and E-cadherin is obviously very complex. ER-negative cell lines are often (but not always, as shown in Fig. 2*B* in MCF10A cells) E-cadherin-negative, and this has recently been analyzed in more detail. The repressor *MTA3* is an estrogen-regulated gene that regulates Snail expression, which in turn represses E-cadherin (9). Thus, in the absence of ER (and *MTA3*), aberrant expression of Snail results in a loss of expression of E-cadherin. Additionally, as shown here, in ER-positive tumors, estrogen can result in down-regulation of E-cadherin expression. How do these findings relate to known clinical data? Not surprisingly, there is little consensus between numerous studies addressing the relationship between hormone receptor status and E-cadherin expression. There have been studies showing a positive (28), a negative (29), or no correlationship (30) between E-cadherin and ER levels. This might, at least in part, be explained by the analysis of "mixed samples," *i.e.*, ER-positive as well as ER-negative samples. As clearly shown by Fujita *et al.* (Fig. 7 in Ref. 9), ER-positive and ER-negative tumors display very different and even opposite correlations between ER, E-cadherin, Snail, and MTA3. We would like to propose that this, at least in part, results from the estrogen-mediated down-regulation of E-cadherin in ER-positive samples. More clinical studies analyzing either only ER-positive or only ER-negative cases are needed to support these models. Potentially, our findings could have clinical impact, because restoration of E-cadherin expression might be an important result of antiestrogen therapy, and, thus, selective estrogen receptor modulators (SERMs) should be tested regarding their effects on E-cadherin expression.

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Insulin-Like Growth Factor-I Inhibits Progesterone Receptor Expression in Breast Cancer Cells via the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Pathway: Progesterone Receptor as a Potential Indicator of Growth Factor Activity in Breast Cancer

XIAOJIANG CUI, PING ZHANG, WANLENG DENG, STEFFI OESTERREICH, YILING LU, GORDON B. MILLS, AND ADRIAN V. LEE

Breast Center (X.C., P.Z., W.D., S.O., A.V.L.), Baylor College of Medicine; and Department of Molecular Therapeutics (Y.L., G.B.M.), University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Although interactions between estrogen and growth factor signaling pathways have been studied extensively, how growth factors and progesterone regulate each other is less clear. In this study, we found that IGF-I sharply lowers progesterone receptor (PR) mRNA and protein levels in breast cancer cells. Other growth factors, such as epidermal growth factor, also showed the same effect. The decrease of PR levels was associated with reduced PR activity. Unlike progestins, IGF-I does not utilize the proteasome for down-regulating PR. Instead, the IGF-I-mediated decrease in PR levels is via an inhibition of PR gene transcription. In addition, the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway was found to be specifically involved in this IGF-I effect. Our data also suggest that the IGF-I down-regulation of PR is not mediated via a reduction of estrogen receptor (ER) levels or activity. First, IGF-I induced ligand-independent ER activity while reducing ER-dependent PR levels. Second, whereas PR and cyclin D1 are both ER up-regulated, IGF-I increased cyclin D1 levels while decreasing PR levels. Third, constitutively active PI3K or Akt induced ER activity but reduced PR levels and activity. Taken together, our data indicate that IGF-I inhibits PR expression in breast cancer cells via the PI3K/Akt/mTOR pathway. Because low or absent PR in primary breast cancer is associated with poor prognosis and response to hormone therapy, our results suggest that low PR status may serve as an indicator of activated growth factor signaling in breast tumor cells, and therefore of an aggressive tumor phenotype and resistance against hormonal therapy. (*Molecular Endocrinology* 17: 575–588, 2003)

THE IGF SYSTEM is composed of a complex interacting network of ligands, ligand binding proteins, and receptors (1). In different cell types, the IGFs can elicit distinct downstream signaling cascades, of which the phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/MAPK pathways are the best studied (2). The IGFs can lead to cell proliferation, survival, and differentiation. As key regulators of cell cycle progression, they also play an important role in malignant transformation and invasion (3, 4). Extensive study has demonstrated that the IGFs are mitogenic and antiapoptotic agents for breast epithelial cells *in vitro* (5) and are crucial for mammary gland development (6). Additionally, numerous lines of evidence have supported a role for the IGFs in breast cancer pathogenesis (7). First, breast cancer cell lines express all of the components required for eliciting a response to the IGFs, and this results in the IGFs being one of the most potent mitogens for breast cancer cells (8). Second, blockade of IGF action *in vitro* and *in vivo* can inhibit breast tumor growth (9, 10). Third, epidemiological study shows that circulating levels of IGF-I predict breast cancer risk (11). Finally, IGF components are expressed in primary breast tumors, and high expression of several of them are associated with poor prognosis (12).

Progesterone is also critically involved in the development of the mammary gland and breast cancer, and

Abbreviations: DCS, Dextran-charcoal-treated serum; DRB, 5, 6-dichlorobenzimidazole riboside; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphatedehydrogenase; HIMOC, 1L-6-hydroxymethyl-*chiro*-inositol-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate; HRG, heregulin; ICI, ICI 182,780; IGF-IR, IGF-I receptor; IMEM, improved MEM zinc option; IRSs, insulin receptor substrates; luc, luciferase; mTOR, the mammalian target of rapamycin; PBST, PBS plus 0.05% Tween-20; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PR, progesterone receptor; PR-A and PR-B, two isoforms of human PR; PRE, progesterone response element; Q-PCR, real-time quantitative RT-PCR; SFM, serum-free medium; tk, thymidine kinase.

its effects are mostly mediated via the progesterone receptor (PR; Refs. 13–15). Mice lacking PR display incomplete mammary ductal branching and failure of lobular-alveolar development (16). Although PR expression is estrogen receptor (ER) dependent (17, 18), some breast cancer cell lines constitutively express high levels of PR independent of estrogens (19). Human PR normally exists in two isoforms (PR-A and PR-B) of 94 and 116 kDa, originating from two PR promoters (20).

Progesterone is considered differentiative in the uterus but proliferative in the normal mammary gland (14). However, progestin inhibition of breast cancer cell growth in tissue culture has been well documented (21–23). High doses of progestins have been used to treat estrogen-mediated mammary carcinomas, even though their antitumor mechanisms are not clear (14). Interestingly, progestins have been found to exert a biphasic regulation of breast cancer cell growth—accelerating cells through the first mitotic cell cycle, then arresting them in G1 of the second cycle. At this stage, the cell cycle progression machinery is poised to restart, as expression of growth factors and their receptors is increased by progestins (21, 22). Thus, it is proposed that progestins are neither inherently growth proliferative or inhibitive, but rather sensitize cells for growth factor and cytokine signals (24, 25).

The presence of PR in breast tumors is an important indicator of likely responsiveness to endocrine agents (13, 26). Approximately two thirds of breast cancers express the ER, some of which are ER positive (ER+)/PR negative (PR−). Their likelihood of response to endocrine therapy drops significantly compared with those that are ER+/PR+. It has also been reported that absence of PR in primary breast tumors is associated with secondary breast cancer in postmenopausal women (27), and absence of PR correlated significantly with a less differentiated phenotype of breast tumors (G1/G2 grading) and the presence of ErbB2/HER2/neu (28). Abnormal expression of ErbB2 and other growth factor receptors is normally associated with more aggressive tumors and a poorer patient prognosis (29, 30). So is there an intrinsic correlation between PR and intensity of growth factor action? We hypothesize that PR status may reflect growth factor function: low or absent PR expression indicates high IGF, epidermal growth factor (EGF), and heregulin (HRG) activities, and this correlation is independent of ER status. This assumption, combined with the fact that progesterone may inhibit breast tumor invasion, might explain why absence of PR is a marker of an aggressive tumor phenotype (27).

Recently, much effort has been directed to the study of the cross-talk between growth factors and ER signaling pathways in breast cancer cells (31), but how growth factors may interact with PR is less well defined. In this study, we have investigated the mechanisms for growth factor regulation of PR in breast cancer using IGF-I and MCF-7 cells because these cells are sensitive to the IGFs, have considerable PR levels, and possess intact PI3K, MAPK, and other common signaling pathways. We find that IGF-I dramatically down-regulates PR through a transcriptional mechanism involving the PI3K pathway, independent of ER activity. Our data provide the first evidence that activation of a growth factor signaling pathway can directly reduce PR levels, and may explain why PR-negative tumors, which possibly have highly active growth factor signaling, poorly respond to endocrine therapy.

RESULTS

IGF-I Inhibits PR Expression

As a first step to investigate the effect of IGF-I on PR in breast cancer cells, we treated MCF-7 cells with IGF-I and then performed Western blot analysis to determine how PR expression was affected. We found that IGF-I treatment caused a dramatic decrease of both PR-A and PR-B protein levels in MCF-7 cells. The dose-response assay using a 24-h time point of treatment showed that maximal reduction of PR protein levels occurred with 10 nM IGF-I (Fig. 1, A and B), which is physiologically relevant to the circulating concentrations of IGF-I in women (11). Much lower concentrations of IGF-I, 0.1 nM, also resulted in an apparent reduction of PR protein levels. From a time course experiment using 10 nM IGF-I, it was found that PR protein levels began to drop after 6 h of IGF-I treatment and continued to decrease with time, whereas the untreated control cells did not display visible down-regulation of PR (Fig. 1, C and D). After 24 h, the sharp fall in PR protein levels stopped, but levels remained suppressed for at least 72 h. We also observed similar results in other PR+ breast cancer cells such as T47D and ZR75 (data not shown). Hence, these data indicate that IGF-I lowers PR levels in breast cancer cells.

To ascertain whether PR activity changed correspondingly with its protein levels after IGF-I treatment, we transiently transfected a progesterone response element (PRE)-luciferase construct into MCF-7 cells. Treatment with the synthetic progestin R5020 caused a more than 100-fold increase in reporter activity, which was almost completely blocked by the antiprogestin RU486 (Fig. 1E). IGF-I pretreatment for 6 h caused a 70% reduction in R5020-induced luciferase activity. This was most probably due to the decrease of PR protein levels elicited by IGF-I, which by itself, at the same time point, had no detectable activation of the reporter activity over the unstimulated control. Hence, these data suggest that IGF-I represses PR transcriptional activity through down-regulation of PR in MCF-7 cells.

Because MCF-7 cells also respond well to other growth factors like EGF and HRG (although response to EGF is not as sensitive as the response to HRG and IGF-I), and these growth factors share similar signaling pathways, we subsequently examined the effect of

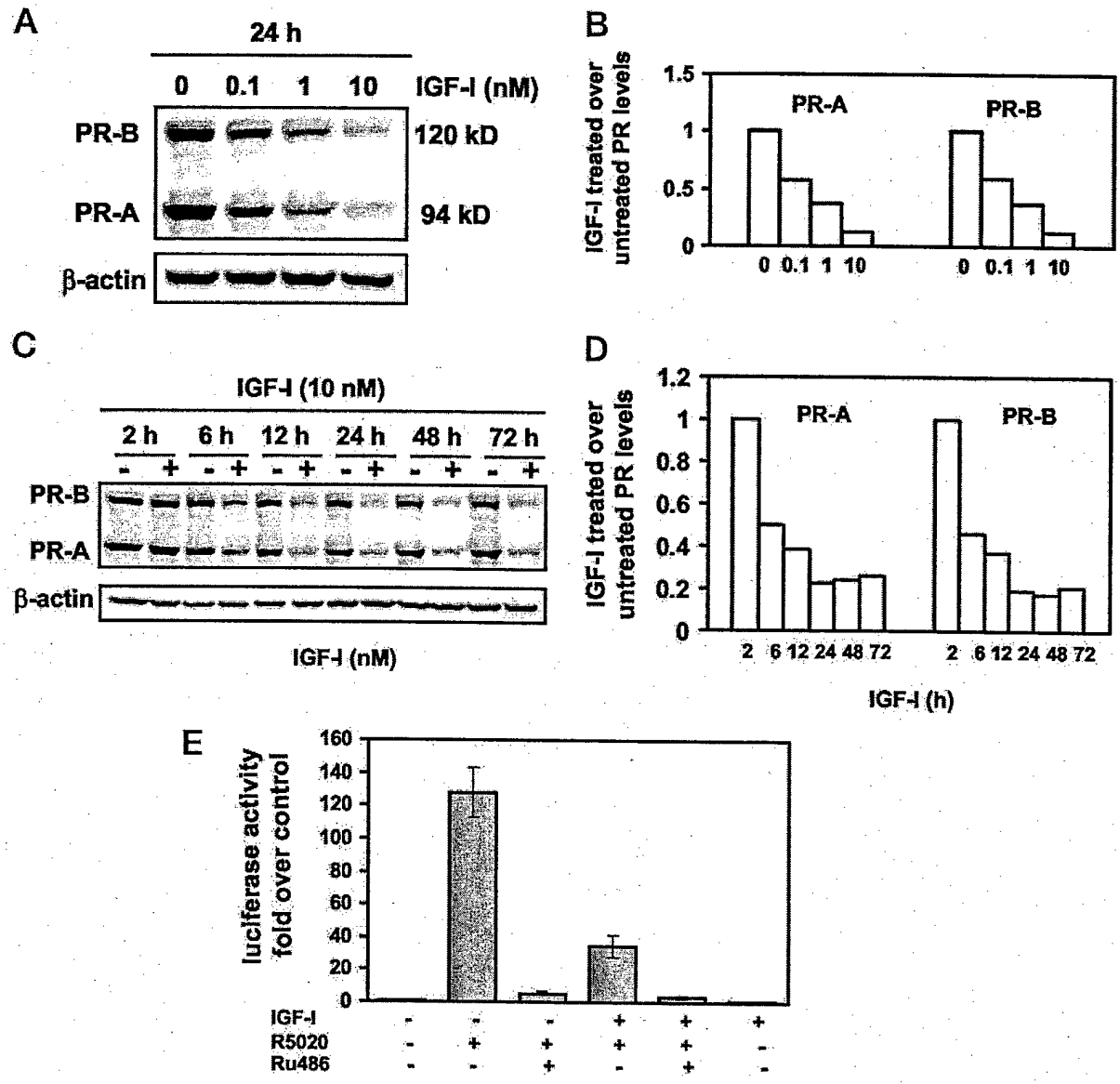

Fig. 1. IGF-I Down-Regulates PR in MCF-7 Cells

A, MCF-7 cells were stimulated for 24 h with increasing concentrations of IGF-I (0.1, 1, 10 nM). Cell lysate (40 μg) was separated by 8% SDS-PAGE and immunoblotted with anti-PR antibody. β-Actin was used as a loading control. B, Graphical representation of data in panel A after densitometry and correction for β-actin expression. PR levels (represented by the densitometric readings) in IGF-I-treated samples were compared with those in the control sample. C, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time. Cell lysates were immunoblotted with anti-PR antibody. D, Graphical representation of data in panel C after densitometry and correction for β-actin expression. PR levels (represented by the densitometric readings) in IGF-I-treated samples were compared with those in the control sample at each time point. E, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μg PRE-tk-luc vector. After treatment with 10 nM IGF-I for 6 h, cells were stimulated with 10 nM R5020, 100 nM RU486, or a combination for 12 h. Then, cells were lysed and luciferase assays were performed. The β-galactosidase expression vector pSV-β-Gal was used as an internal transfection control. Values are means ± SE of three independent experiments, each in duplicate.

EGF, HRG, and insulin on PR expression using immunoblotting. As a control, we treated cells with R5020 and found the characteristic upward protein mobility shift (due to phosphorylation) and decrease in PR levels (due to proteasomal degradation—see Fig. 2B) as reported previously (32). Not surprisingly, HRG and EGF at similar concentrations to that of IGF-I also markedly down-regulated PR (Fig. 2A), but insulin required 100-fold higher concentrations than IGF-I to achieve the same PR reduction. This suggests that the reduction of PR expression by IGF-I is a common effect shared by other growth factors in MCF-7 cells.

As reported previously, IGF-I can induce extensive phosphorylation of insulin receptor substrates (IRSs),

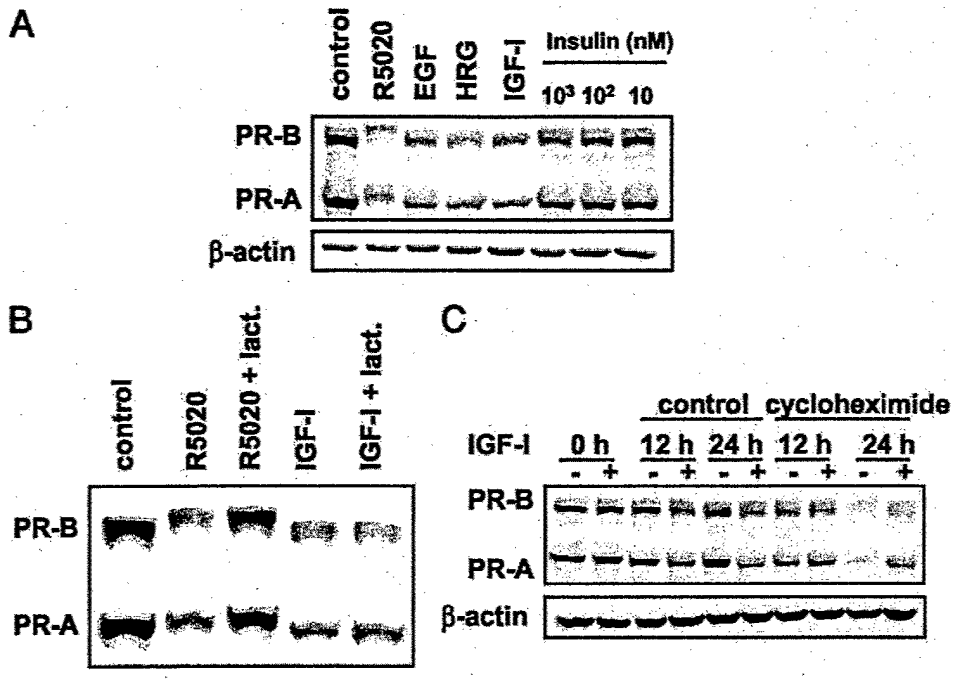

Fig. 2. IGF-I, EGF, HRG, and Insulin Down-Regulate PR by a Different Mechanism than R5020

A, MCF-7 cells were stimulated for 24 h with 20 nM EGF, 10 nM HRG, 10 nM IGF-I, increasing concentrations of insulin (10, 10^2, 10^3 nM), or 10 nM R5020. Cell lysates were immunoblotted with anti-PR antibody. β-Actin was used as a loading control. B, Cells were first treated with the 26S proteasome inhibitor lactacystin (10 μM) for 30 min before IGF-I and R5020 stimulation in the presence of lactacystin for 12 h. Cell lysates were immunoblotted with anti-PR antibody. C, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time in the presence of the translation inhibitor cycloheximide (10 μg/ml) after preincubation with cycloheximide for 30 min. Cell lysates were immunoblotted with anti-PR antibody. β-Actin was used as a loading control. This figure is representative of three independent experiments.

which leads to an upward protein mobility shift on SDS-PAGE gels and decreased IRSs levels via proteasomal degradation (33). Similarly, progestins cause phosphorylation and a mobility up-shift of PR, and they decrease PR levels via proteasomal degradation (32). Interestingly, IGF stimulation did not lead to a mobility shift of PR on SDS-PAGE gels, unlike the progestin R5020 (Fig. 2, A and B). Thus, the mechanism by which IGF-I down-regulates PR in breast cancer cells may be different from the 26S proteasome degradation pathway. This was confirmed by the inability of lactacystin, a 26S proteasome inhibitor, to block the decrease of PR protein levels by IGF-I (Fig. 2B). To further investigate how IGF-I affects PR protein stability, we used the translation inhibitor cycloheximide. Western blot analysis demonstrated that, in contrast to noncycloheximide conditions, PR protein levels were not lowered by IGF-I treatment compared with non-IGF treatment under cycloheximide conditions (Fig. 2C, see 12-h and 24-h lanes). This indicates that IGF-I does not impede PR protein stability, which is consistent with the result from the proteasome inhibitor assay (Fig. 2B).

IGF-I Represses PR Transcription

To investigate whether IGF-I suppresses PR protein levels by inhibiting PR transcription, we conducted real-time quantitative RT-PCR (Q-PCR) to detect changes of PR mRNA concentrations in MCF-7 cells after IGF-I treatment. Because more than five PR mRNA transcripts with a wide range of sizes have been found in breast cancer cells and tumors, and it has not been clarified which codes for PR-A, PR-B, or both (20, 34), we chose primers and the fluorescent probe located near the 3′ end of the PR coding region so as to detect all PR transcripts. We found that total PR mRNA transcript levels gradually decreased during the 24-h time period (Fig. 3A), which can probably be attributed to the fact that PR is ER dependent and estradiol is depleted in the serum-free medium (SFM) that was used in our cell stimulation experiments. However, compared with controls, PR mRNA levels dropped dramatically after 2 h of IGF-I treatment and continued to decrease with time, which was consistent with the change of PR protein levels in the IGF-I time course experiment (Fig. 1B). Interestingly, PR protein levels did not show a detectable drop at the 2-h time point, suggesting that the repression of PR mRNA levels by IGF-I occurred earlier than the reduction of PR protein levels. At any selected time point, PR mRNA levels under IGF-I-treated conditions were clearly lower than untreated controls. The decrease of PR mRNA levels by IGF-I was confirmed by Northern blotting using a probe at the 3′ end of the PR coding

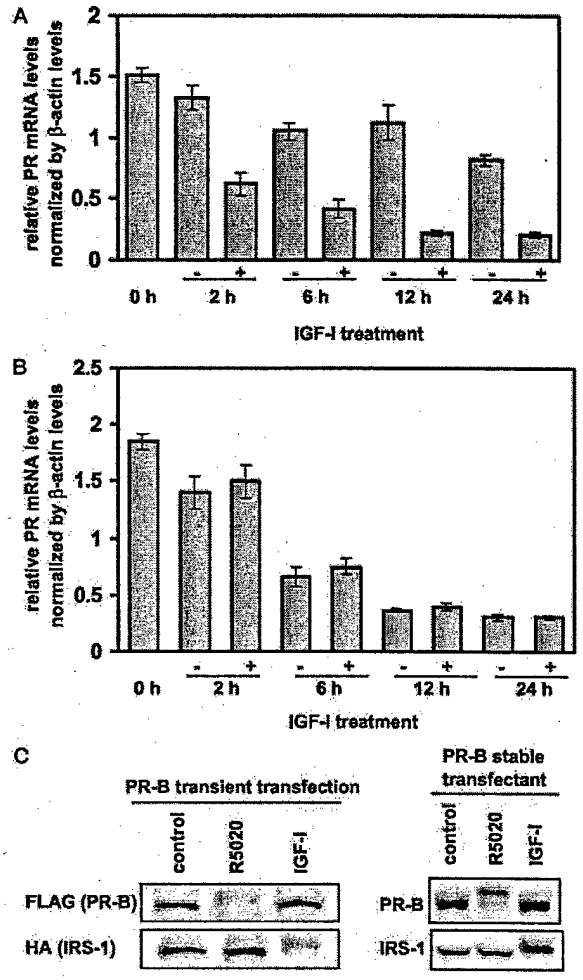

Fig. 3. IGF-I Down-Regulates PR mRNA Levels in MCF-7 Cells

A, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time. Total RNA was isolated and PR mRNA levels measured by Q-PCR. PR mRNA levels in each sample were calculated from a standard curve and normalized using β-actin mRNA levels. B, MCF-7 cells were stimulated with 10 nM IGF-I or vehicle for increasing periods of time in the presence of the transcription inhibitor DRB (50 μM) after preincubation with DRB for 30 min. Total RNA was isolated and Q-PCR was performed. C, FLAG-PR-B and HA-IRS-1 constructs were transiently cotransfected into MCF-7 cells. After 18 h, cells were stimulated with 10 nM IGF-I or 10 nM R5020 for 24 h. Similarly, stable transfectants of PR-B were stimulated with 10 nM IGF-I or 10 nM R5020 for 24 h. Cell lysates were immunoblotted with anti-FLAG, anti-HA, anti-IRS-1, and anti-PR antibodies. IRS-1 mobility shift and down-regulation was used as a marker for IGF-I responsiveness. This figure is representative of two independent experiments.

region (data not shown). These results indicate that IGF-I represses PR mRNA levels in breast cancer cells.

To determine whether posttranscriptional mechanisms are also involved in the IGF-I down-regulation of PR mRNA, we preincubated MCF-7 cells with the transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB), and then stimulated the cells with IGF-I in the presence of DRB. Q-PCR showed that in the presence of DRB, PR mRNA levels dropped due to RNA degradation (Fig. 3B). However, in contrast to non-DRB conditions (Fig. 3A), PR mRNA levels under DRB conditions were not lowered by IGF-I compared with non-IGF-I treatment. The same result was confirmed by Northern blotting (data not shown). This suggests that IGF-I does not impair PR mRNA stability.

To confirm that IGF-I down-regulates PR through regulation of PR promoter activity, we transiently transfected a FLAG epitope-tagged PR-B cDNA and an HA epitope-tagged IRS-1 cDNA, both of which were driven by the cytomegalovirus (CMV) promoter, into MCF-7 cells. As expected, immunoblotting showed that IGF-I treatment did not reduce the exogenous PR protein levels that were generated from a heterologous promoter (CMV), whereas it did lower the exogenous IRS-1 levels and cause its mobility up-shift (Fig. 3C). As a control, the exogenous PR protein could still be regulated normally by R5020, *i.e.* PR protein levels were decreased and PR protein mobility was shifted upward. Furthermore, we stably transfected the PR-B cDNA into a specifically selected MCF-7 cell subline, which does not have detectable ER and PR but still responds to IGF-I (35). Similar to the transient transfection, IGF-I did not decrease PR-B levels in the stable transfectants but altered the endogenous IRS-1 levels and mobility (Fig. 3C). Then we transiently transfected a PRE-luciferase construct into these PR-B stable transfectants. Opposite to the data in regular MCF-7 cells, IGF-I could not inhibit the R5020-induced luciferase activity (data not shown).

In summary, these data show that IGF-I down-regulates PR mRNA transcription, and this is dependent upon the original PR promoter context.

IGF-I Regulates PR Expression through the PI3K/Akt/Mammalian Target of Rapamycin (mTOR) Pathway

The transmission of IGF signals in breast cancer cells involves several well-characterized signaling cascades downstream of IGF-I receptor (IGF-IR), including the PI3K/Akt and Ras/MAPK pathways (2). To identify the signaling pathway implicated in the IGF-I down-regulation of PR, we used a series of potent signaling inhibitors against Akt, MEK, mTOR, p38 MAPK, PI3K, protein kinase A (PKA), and protein kinase C (PKC). These inhibitors have been widely used in signal transduction research including our own previous studies (33). MCF-7 cells were first preincubated with these inhibitors and then stimulated with IGF-I in the presence of the inhibitors. Western blot analysis showed that blockade of Akt with 10 μM 1L-6- hydroxymethyl-*chiro*-inositol- (R)-2-O-methyl-3-O-octadecylcarbonate (HIMOC), of mTOR with 40 nM rapamycin, or of PI3K with 20 μM LY294002, rescued IGF-I down-regulated PR to the control level (Fig. 4A). In contrast, the MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, the PKA inhibitor H-89, and the PKC inhibitor GF109203X did not prevent IGF-I re-

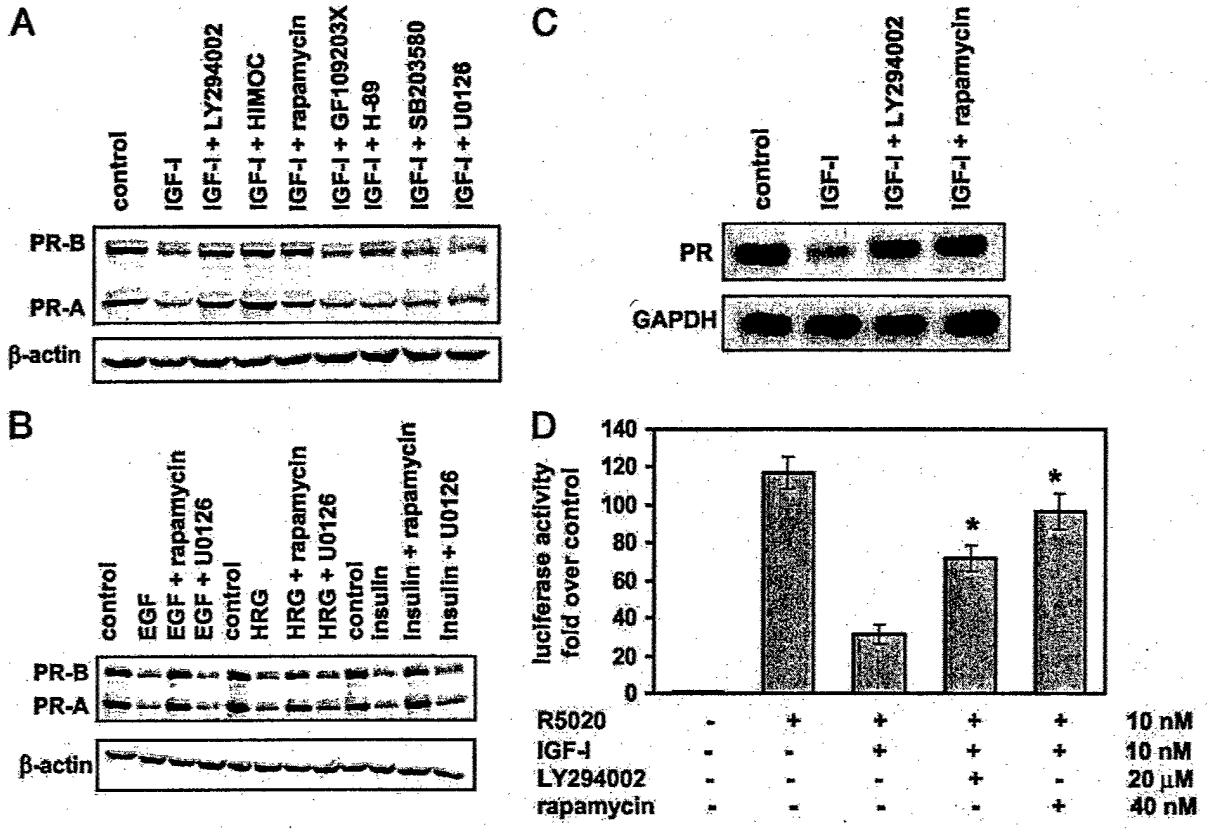

Fig. 4. The PI3K Inhibitor LY294002 and the mTOR Inhibitor Rapamycin Prevent IGF-I Down-Regulation of PR

A, MCF-7 cells were stimulated with 10 nM IGF-I for 24 h in the presence of the inhibitors after preincubation with 20 μM LY294002 (PI3K), 10 μM HIMOC (Akt), 40 nM rapamycin (mTOR), 5 μM GF109203X (PKC), 10 μM H-89 (PKA), 10 μM SB203580 (p38 MAPK), or 5 μM U0126 (MEK) for 30 min. Cell lysates were immunoblotted with anti-PR antibody. β-Actin was used as a loading control. B, MCF-7 cells were stimulated with 20 nM EGF, 10 nM HRG, or 1 μM insulin for 24 h in the presence of the inhibitors of mTOR and MEK. Cell lysates were immunoblotted with anti-PR antibody. β-Actin was used as a loading control. C, MCF-7 cells were stimulated with 10 nM IGF-I in the presence of the inhibitors for 12 h after preincubation with 20 μM LY294002 or 40 nM rapamycin for 30 min. Total RNA was isolated and RT-PCR was conducted. PCR product was visualized with ethidium bromide staining under UV light. GAPDH was used as an internal control for RT-PCR. D, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μg PRE-tk-luc vector. The cells were preincubated with LY294002 and rapamycin for 30 min before IGF-I treatment for 6 h followed by R5020 stimulation for 12 h. Then, cells were lysed and luciferase assays were performed. pSV-β-Gal was used as an internal transfection control. Values are means ± SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with IGF-I plus R5020 treatment.

duction of PR in MCF-7 cells. These results were confirmed using other common inhibitors against the same target kinases such as the MEK inhibitor PD98059, the PI3K inhibitor wortmannin, and the PKC inhibitor GO6983 (data not shown). As PI3K, Akt, and mTOR form a sequential signaling cascade activated by the IGF-IR (36), the IGF-I effect on PR appears to be specifically elicited via the PI3K/Akt/mTOR pathway. Not surprisingly, the same signaling pathway was found to be involved in the EGF, HRG, and insulin down-regulation of PR (Fig. 4B). Because IGF-I represses PR at the transcription level, we confirmed by RT-PCR that inhibitors of PI3K or mTOR abolished the decrease of PR mRNA by IGF-I (Fig. 4C).

As shown previously (Fig. 1E), IGF-I down-regulates R5020-induced PR transcriptional activity. To assess the role of PI3K and mTOR in this event, we transiently transfected a PRE-luciferase construct into MCF-7 cells. Subsequent stimulation experiments using R5020, IGF-I, and the PI3K and mTOR inhibitors showed that the inhibitors impaired IGF-I reduction of R5020-induced luciferase activity (Fig. 4D). Noticeably, luciferase activity did not fully recover, unlike the PR protein levels (Fig. 4A). We speculate that the toxicity of these inhibitors to transfected cells and to the transcription machinery on the PRE promoter may contribute to their partial effect.

To confirm that the PI3K/Akt/mTOR pathway is involved in IGF-I down-regulation of PR, we used MCF-7 cells stably transfected with either a kinase-defective Akt (KD-Akt) containing a K179M substitution or a constitutively active Akt (myr-Akt) containing a myristoylation membrane-targeting sequence. Western blot analysis showed that overexpression of KD-Akt moderately elevated PR levels in MCF-7 cells in SFM, whereas overexpression of myr-Akt dramatically de-

creased PR levels (Fig. 5A). Interestingly, ERα protein levels were not different in the parental and transfected MCF-7 cells. As expected, mTOR phosphorylation was attenuated in KD-Akt cells but enhanced in myr-Akt cells (Fig. 5A). These data are consistent with Akt having a role in IGF-I down-regulation of PR in breast cancer cells.

To examine how ER regulates PR in both stable transfectants, we treated the cells with estradiol. As shown in Fig. 5B, 10 nM estradiol induced PR expression over the basal control levels in both KD-Akt and myr-Akt cells, suggesting that ER functions properly in both cell lines and that the effect of mutant Akt on PR is not mediated by influencing ER. Interestingly, LY294002 pretreatment displayed a stronger effect in blocking the IGF-I effect in KD-Akt transfectants than in myr-Akt transfectants, although rapamycin had a similar effect in both cells (Fig. 5B). This can be explained by the fact that Akt is downstream of PI3K and upstream of mTOR, and that myr-Akt activity is largely independent of growth factor and PI3K activation. Taken together, our data demonstrate that the PI3K/Akt/mTOR pathway uniquely mediates the alteration of PR expression elicited by IGF-I in breast cancer cells.

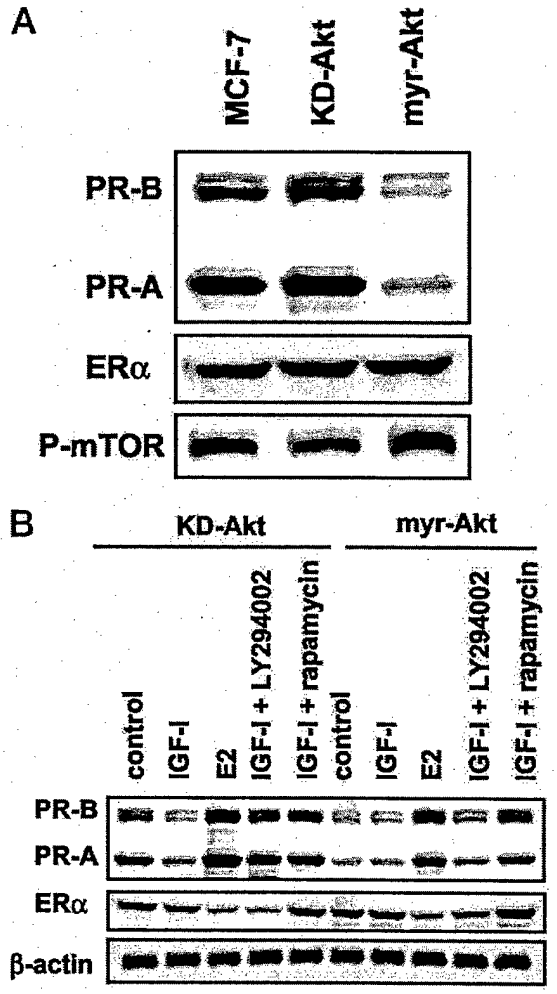

Fig. 5. Expression of KD-Akt and myr-Akt Alters PR Levels in MCF-7 Cells

A, Cell lysates from stable transfectants of KD-Akt and myr-Akt, and parental MCF-7 cells grown in SFM for 48 h were immunoblotted with anti-PR, anti-ERα, and anti-phospho-mTOR antibodies. B, KD-Akt and myr-Akt transfectants were stimulated for 24 h with 10 nM estradiol or 10 nM IGF-I in the presence of the inhibitors after preincubation with 20 μM LY294002 or 40 nM rapamycin for 30 min. Cell lysates were immunoblotted with anti-PR and anti-ERα antibodies. β-Actin was used as a loading control. This figure is representative of three independent experiments.

IGF-I Down-Regulation of PR Is Independent of ER Activity

It has been reported previously that IGF-I down-regulates ER levels in breast cancer cells (37, 38), which we also found (data not shown). Accordingly, this raised a question as to whether the IGF-I effect on PR was actually caused by the IGF-I effect on ER. This intriguing enigma prompted us to explore if there is a correlation between ER and PR expression regulated by IGF-I.

Initially, we had three pieces of evidence to suggest that the down-regulations of PR and ER are two independent events triggered by IGF-I. First, PR expression in T47D breast cancer cells, which is independent of ER (19), was also significantly reduced by IGF-I (data not shown). Second, as shown in Fig. 5, PR levels were markedly lower in myr-Akt stable transfectants than in KD-Akt cells, even though ER levels, and ER's ability to induce PR, were similar in both cells. Third, PR levels were remarkably lower when MCF-7 cells were grown in dextran-charcoal-treated serum (DCS: estradiol and other steroid hormones removed, but growth factors maintained) medium than in SFM, whereas ER levels were constant in both media (data not shown). Thus, to further test this separation of effects, we performed the following experiments.

Previous reports have shown that IGF-I can cause ligand-independent activation of ER via alteration of ER phosphorylation status in breast cancer and other cells, and even *in vivo* (39–41). To test this in our experimental system, we transiently transfected an estrogen response element (ERE)-luciferase vector into MCF-7 cells, which were then stimulated with estradiol, IGF-I, or the antiestrogen ICI 182,780 (ICI). As shown in Fig. 6A, luciferase activity was significantly increased by estradiol or IGF-I and was further elevated by the two together, whereas ICI blocked the effect of both estradiol and IGF-I, suggesting that the IGF-I-induced luciferase activity is mediated by ER and accordingly that IGF-I can directly induce the transcriptional activity of ER. Surprisingly, Western blot analysis showed that estradiol and IGF-I cotreatment severely attenuated estradiol-induced PR levels, which dropped even lower than that of the unstimulated control (Fig. 6B). This suggests that IGF-I may directly repress PR expression, even while it activates ER, and that this repression is dominant over ER ac-

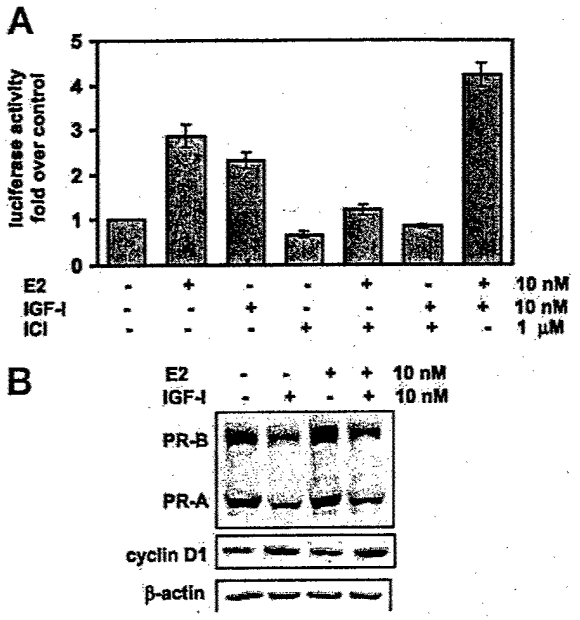

Fig. 6. IGF-I Induces Ligand-Independent ER Activity While Reducing Estradiol-Induced PR Expression

A, MCF-7 cells in six-well dishes were transiently transfected with 0.2 μg ERE-tk-luc vector. After stimulation with 10 nM IGF-I, 10 nM estradiol, 1 μM ICI, or their combination for 12 h, cells were lysed and luciferase assays were performed. pSV-β-Gal was used as an internal transfection control. Values are means ± SE of three independent experiments, each in duplicate. B, MCF-7 cells were stimulated with 10 nM IGF-I, 10 nM estradiol, or their combination for 12 h. Cell lysates were prepared and immunoblotted with anti-PR and anti-cyclin D1 antibodies. β-Actin was used as a loading control.

tivity on PR. In contrast to PR, expression of cyclin D1, which is also ER dependent (42), was increased by estradiol, IGF-I, and the two together (43, 44). Thus, IGF-I has opposite effects on ER-inducible PR and ER-inducible cyclin D1 expression, even though it activates ER transcriptional activity.

To elucidate the difference between the IGF-I effects on ER activity and PR levels, we transiently cotransfected ERE-luciferase with myr-Akt or a constitutively active PI3K catalytic subunit p110 (myr-PI3K) into MCF-7 cells. As shown in Fig. 7A, estradiol or IGF-I treatment caused a 2- to 3-fold increase of the reporter activity over the nontreated control, whereas overexpression of myr-Akt or myr-PI3K led to a similar or higher increase of the luciferase activity in the absence of estradiol. This was due to the ligand-independent activation of ER by Akt and PI3K (PI3K utilizes both Akt-dependent and Akt-independent pathways) (45, 46). On the other hand, when we transiently cotransfected PRE-luciferase with myr-Akt or myr-PI3K into MCF-7 cells, either IGF-I or overexpression of myr-Akt or myr-PI3K inhibited the R5020-induced luciferase activity (Fig. 7B). Thus, myr-Akt and myr-PI3K can mimic the effect of IGF-I in suppressing PR activity through down-regulation of PR. Taken together, these data indicate that IGF-I regulates ER activity and ER-dependent PR levels in opposite ways, and that IGF-I down-regulation of PR expression is independent of ER (Fig. 8).

DISCUSSION

PR is an important factor in mammary gland and breast cancer development. Because PR is a highly ER-dependent gene, regulation of PR expression by other proteins and growth factors has been naturally regarded as indirect and attributed to changes of ER status. Here, we provide evidence showing that IGF-I down-regulates PR in breast cancer cells irrespective of ER activity through a transcriptional mechanism involving the PI3K/Akt/mTOR signaling pathway (Fig. 8).

In this study, we found that EGF, HRG, and insulin can also sharply reduce PR levels. An obvious common feature between IGF-I and these growth factors is that they all can activate the PI3K/Akt signaling cascade in breast cancer cells, which explains why they all display the same effect on PR expression. One could speculate that other growth factors like PDGF, which also activates the PI3K/Akt pathway through its receptors, may likewise down-regulate PR in breast cancer cells that express PDGF receptors (47). But why does insulin require much higher doses to achieve the same effect as IGF-I even though MCF-7 cells have relatively high levels of insulin receptor (48)? We found that IGF-I is more potent than insulin at the same concentration (10 nM) to induce phosphorylation of IRS-1 and Akt, an indicator of activated PI3K/Akt signaling (Ref. 33 and data not shown). Thus, high concentrations of insulin may be necessary for insulin to act through IGF-IR to decrease PR levels in MCF-7 cells because insulin has a relatively low affinity to IGF-IR (33, 49). Alternatively, high doses of insulin may simply enhance the insulin receptor-mediated PI3K/Akt signaling pathway.

It is a long-held rule of thumb that optimal estradiol stimulation of PR occurs in medium supplemented with 5% DCS (50). This may be attributed to the fact that DCS medium contains large amount of growth factors and consequently suppresses PR to barely detectable levels, whereas the basal level of PR is much higher in SFM. Hence, the fold-induction of PR with estradiol is seemingly more dramatic in DCS medium. This is perhaps also the reason behind the previous conflicting reports in which PR was used as a reporter gene monitoring ER activity in the study of IGF-I regulation of ER. Clayton *et al.* (37) showed that insulin and IGF-I considerably impaired the estradiol induction of PR mRNA in SFM, which is in line with our result. In another study that showed that IGF-I had a potentiating effect on the estradiol induction of PR mRNA (38), the cells were grown in DCS medium for 2 d. In this system, because PR transcription was already suppressed by growth factors, the observed IGF-I effect might actually indicate a change in PR

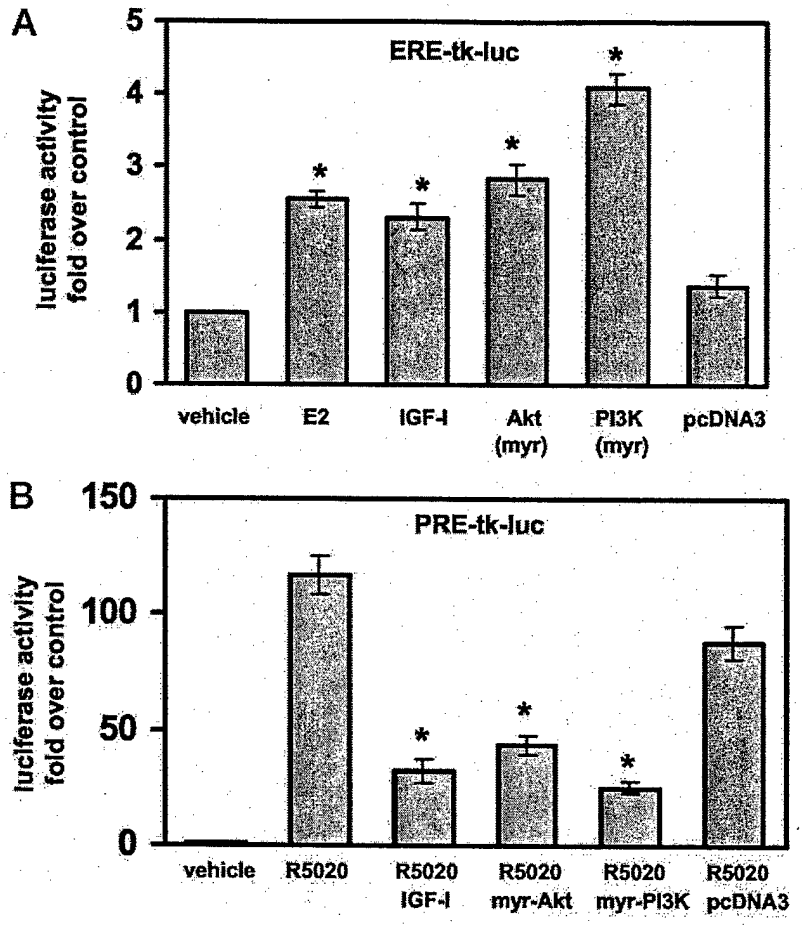

Fig. 7. Myr-Akt and myr-PI3K Mimic the IGF-I Effect on Ligand-Independent ER Activity and R5020-Induced PR Activity

A, MCF-7 cells in six-well dishes were transiently cotransfected with 0.2 μg ERE-tk-luc and 0.5 μg myr-Akt, myr-PI3K, or empty pcDNA3 vector. ERE-tk-luc-only transfected cells were stimulated with 10 nM estradiol or 10 nM IGF-I and were compared with nonstimulated ERE-tk-luc plus myr-Akt or myr-PI3K cotransfected cells in luciferase assay. Values are means ± SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with pcDNA3 transfection. B, MCF-7 cells in six-well dishes were transiently cotransfected with 0.2 μg PRE-tk-luc and 0.5 μg myr-Akt, myr-PI3K or pcDNA3.1 vectors. Cells were treated with R5020 to induce PR activity. PRE-tk-luc-only transfected cells were stimulated with 10 nM R5020 or 10 nM IGF-I plus 10 nM R5020. Values are means ± SE of three independent experiments, each in duplicate. *, $P < 0.005$ as compared with pcDNA3 transfection.

mRNA and/or protein stability. Previously, Cho *et al.* (50) reported that IGF-I does not affect PR transcription but elevates PR protein levels in MCF-7 cells under SFM conditions. The discrepancy between their and our results may be due to the different cell culture conditions before IGF-I stimulation. In our study, before IGF-I treatment, cells were starved for 16 h after being switched to SFM from regular culture medium, whereas there was no delay of IGF-I treatment after the change to SFM from DCS medium in their studies.

Recently, evidence is emerging to support our conclusion that IGF-I and other growth factors inhibit PR expression. First, it was found that overexpression of ER in a specifically selected ER−/PR− MCF-7 breast cancer cell subline did not restore PR expression, although ER did restore cyclin D1, IRS-1, and IGF-IR levels (35). These cells were grown in DCS medium, which might completely suppress PR expression after long-term cell culture. Similarly, it was reported that in antiestrogen-resistant MCF-7 cells generated by continuous culture of the PR+ parental cells in antiestrogen-supplemented DCS medium, EGF receptor signaling was enhanced, whereas PR levels diminished (51). Interestingly, replacement of antiestrogen by estradiol failed to induce PR, whereas expression of other estrogen-responsive genes was significantly elevated. Conceivably, DCS medium plus enhanced EGF receptor signaling may permanently silence PR expression. In another interesting report, the mammary epithelial cells that retain stem/progenitor cell characteristics were found to lack PR (52). These cells rely on growth factors to continuously proliferate. Recently, in a study that confirmed that ER+/PR+ breast cancer patients respond better to hormonal therapy than ER+/PR− patients, Dowsett *et al.* (53) found that among ER+/PR− samples 25% are HER2+, com-

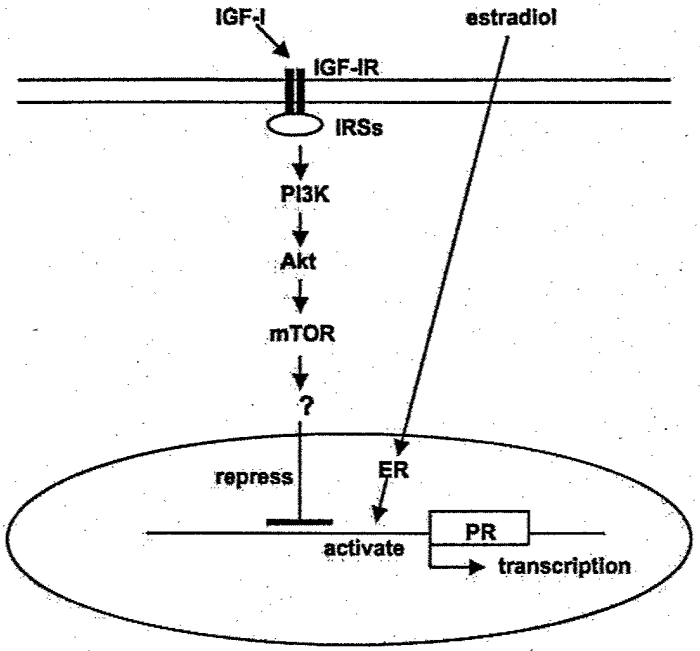

Fig. 8. Illustration of the IGF-I Signaling Pathway Repressing PR Expression

pared with 10% HER2+ among ER+/PR+. Moreover, HER2+/PR− patients responded much worse than HER2−/PR+ in hormonal therapy, again suggesting that PR status may reflect HER2 signaling.

Our result indicates that the PI3K/Akt/mTOR pathway is responsible for the IGF-I down-regulation of PR. In a relevant report, Shen *et al.* (54) showed that, although overexpression of MEKK1, through p42 and p44 MAPK, increased R5020-induced PR activity, it alone could not down-regulate PR. Thus, PI3K/Akt and Ras/MAPK pathways may play distinct roles in regulating PR activity in breast cancer cells. However, we only observed IGF-I down-regulation of PR despite IGF-I's ability to trigger both PI3K/Akt and Ras/MAPK pathways in MCF-7 and other breast cancer cells. This may be due to the finding that Ras can also bind to and activate PI3K (55), and the PI3K/Akt pathway is very potent in eliciting IGF-I signals in MCF-7 cells. Hence, the PI3K/Akt pathway is dominant in IGF-I regulation of PR. Interestingly, the PI3K/Akt/mTOR pathway is also involved in the IGF-I reduction of IRSs (33). The former event is mediated by a transcriptional mechanism, whereas the latter is via the 26S proteasome pathway for protein degradation. Different downstream proteins of mTOR may account for this seeming dilemma—although p70 S6 kinase is a typical target for mTOR, other kinases or signaling proteins may also function downstream of mTOR. For example, it has been shown that insulin regulation of IGFBP-1 gene expression is dependent on mTOR but independent of p70 S6 kinase activity (56). We hypothesize that mTOR may directly or indirectly activate a PR transcription repressor or inactivate a transcription activator to mediate the IGF-I effect on PR expression (Fig. 8).

The ER and PR status in breast cancer is highly correlated with the response to endocrine therapy. Key areas of study in breast cancer are those mechanisms that regulate ER and PR expression. The loss of PR gene expression has been attributed to loss of heterozygosity, loss of ER function, and methylation of a CpG island in the PR promoter (26). Our data provide implications for another theory, in which potent growth factor signaling, especially PI3K/Akt, may contribute to the PR down-regulation. This is reflected clinically with reduced PR levels in breast tumors with HER2 amplification (53). Because the IGF-I effect involves a transcriptional mechanism, whether IGF-I increases methylation of PR promoter and accordingly silences PR expression remains an intriguing question.

Low or absent PR in primary breast cancer is associated with faster disease progression and poor response to hormonal therapy. Our results suggest that low PR may be serving as an indicator of activated growth factor signaling in breast tumor cells, and therefore of an aggressive tumor phenotype and resistance against hormonal therapy.

MATERIALS AND METHODS

Materials

All general materials and chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. IGF-I was purchased from GroPep Pty. Ltd. (Adelaide, Australia). ICI was a kind gift from Zeneca Pharmaceuticals (Macclesfield, UK). The inhibitors GF109203X, H-89, lactacystin, LY294002, HIMOC, rapamycin, SB203580, and U0126 were from Calbiochem (La Jolla, CA). All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise

stated. [^{32}P]deoxy-CTP (3000 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA).

Cell Culture and Plasmids

MCF-7 cells have been maintained in our laboratory for many years (57). Cells were routinely maintained in improved MEM zinc option (IMEM) supplemented with 5% fetal bovine serum, 2 mM glutamine, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 10 μg/ml insulin. SFM consists of IMEM without phenol red plus 10 mM HEPES (pH 7.4), 1 μg/ml of transferrin, 1 μg/ml of fibronectin, 2 mM glutamine, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and trace elements (BioSource Technologies, Inc., Camarillo, CA). Cells were kept at 37 C in a humidified incubator with 5% CO_2. The estrogen-responsive reporter plasmid ERE-tk-luc contains a single consensus ERE upstream of a minimal thymidine kinase (tk) promoter and the luciferase (luc) gene (40). The PRE-tk-luc was constructed in the same way. The expression vectors for constitutively active myr-Akt and myr-PI3K were described elsewhere (58), and were gifts from Dr. Thomas Franke and Dr. Anke Klippel. The murine myr-Akt and myr-PI3K (p110α) each have a myristoylation signal at the N terminus to target the protein to the membrane.

Cell Stimulation and Lysis

Cells were plated at a density of 1.5×10^6 per 6-cm-diameter dish (Becton Dickinson and Co., Lincoln Park, NJ) and allowed to grow for 48 h. Then the medium was changed to SFM, and 16 h later, the cells were stimulated with 10 nM IGF-I, 20 nM EGF, 10 nM HRG, or insulin at 10, 100, or 10^3 nM for different periods of time. For experiments using inhibitors, cells were first preincubated separately with GF109203X (5 μM), H-89 (10 μM), lactacystin (10 μM), LY294002 (20 μM), a HIMOC (10 μM), rapamycin (40 nM), SB203580 (10 μM), and U0126 (5 μM) for 30 min before stimulation with EGF, HRG, IGF-I, and insulin in the presence of an inhibitor as described previously (33). For the translation inhibitor cycloheximide (10 μg/ml), the same procedure was followed. Control cells were incubated with a similar concentration of the vehicle dimethylsulfoxide alone. After stimulation, cells were washed twice with ice-cold PBS and then lysed in 200 μl of lysis buffer, which contained 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 10% glycerol, and a fresh protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Cells were left on ice for 30 min, and then the cell lysate was clarified by centrifugation at 14,000 × *g* for 15 min at 4 C and stored at −20 C. Protein concentration of the supernatant was measured by bicinchoninic acid assay in accordance with the manufacturer's instruction manual (Pierce Chemical Co., Rockford, IL).

Immunoblotting

Total protein (40 μg) was resuspended in denaturing sample loading buffer (3% dithiothreitol; 0.1 M Tris-HCl, pH 6.8; 4% sodium dodecyl sulfate; 0.2% bromophenol blue; 20% glycerol), separated by 8% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane overnight at 4 C. The remaining steps were all performed at room temperature. The membrane was blocked with PBS plus 0.05% Tween-20 (PBST) containing 5% nonfat milk for 1 h and followed by incubation with a 1:1000 dilution of anti-ER (Novocastra Laboratories, Newcastle upon Tyne, UK), anti-FLAG (Sigma), anti-HA (Covance Laboratories, Inc., Richmond, CA), anti-IRS-1 (Upstate Biotechnology, Lake Placid, NY), anti-PR (DAKO Corp., Carpinteria, CA), anti-β-actin (Upstate Biotechnology), and anti-cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies in blocking solution for 1–2 h. For phospho-mTOR detection, the membrane was first washed three times for 5 min each with PBST and then incubated with a 1:1000 dilution of anti-phospho-mTOR antibody (Cell Signaling, Beverly, MA) in PBST. Subsequently, the membrane was again washed three times for 5 min each with PBST and then incubated with a horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at a dilution of 1:4000 in blocking solution. After the membrane was washed three times for 5 min each with PBST, bands were visualized by enhanced chemiluminescence according to the manufacturer's protocols (Pierce Chemical Co.).

RNA Blotting and RT-PCR

Cells were plated at a density of 5×10^6 per 10-cm-diameter dish and allowed to grow for 48 h. Then the medium was changed to SFM. After 16 h starvation, the cells were stimulated with IGF-I for an indicated time period. When the transcription inhibitor DRB (50 μM) and the signaling inhibitors LY294002 and rapamycin were used, cells were first preincubated with them for 30 min before IGF-I stimulation. Total RNA was prepared with RNeasy Midi kit (QIAGEN, Valencia, CA) according to the instruction manual. RNA integrity was checked by separation on a 1% agarose gel. For PCR of the 430-bp PR gene fragment, a pair of primers (5′-CAGTGGGCAGATGCTGTATTTTGC-3′, 5′-GTGCAGCAATAACTTCAGACATC-3′) was designed toward the 3′ end of the PR coding region. Another pair of primers (5′-GGCTCTCCAGAACATCATCCCTGC-3′, 5′-GGGTGTCGCTGTTGAAGTCAGAGG-3′) was used in PCR of the 299 bp glyceraldehyde-3-phosphatedehydrogenase (GAPDH) gene fragment (59). In the RT-PCR experiment, total RNA (2 μg) was used to produce cDNA with Superscript II reverse transcriptase (Invitrogen) in a 20-μl volume. Then, 1 μl from the cDNA synthesis reaction was added to PCR mixture, and PCR amplification was performed with PR and GAPDH primers with an annealing temperature at 60 C and 30 cycles. Products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis.

Q-PCR

Basically, reverse transcriptions of PR mRNA were performed in 96-well optical plates (PE Applied Biosystems, Foster City, CA) using Superscript II reverse transcriptase. All RNA samples were first treated with deoxyribonuclease I to eliminate residual genomic DNA. The reverse primer (5′-GGCTTAGGGCTTGGCTTTC-3′) is at the 3′ end of the PR coding region. Total RNA of 100 ng in a 10-μl reaction volume was added to each well. The plates were incubated at 50 C for 30 min followed by 10 min at 72 C. Then real-time quantitative TaqMan PCR of PR cDNAs was conducted using a PR-specific double fluorescence-labeled probe (5′-TCCCACAGCCATTGGGCGTTC-3′) in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). ROX was used as a reference dye. The PCR mixture also contains 300 nM each of the forward primer (5′-GAGCACTGGATGCTGTTGCT-3′) and the reverse primer. The plates were incubated at 94 C for 1 min, followed by 40 cycles at 94 C for 12 sec and 60 C for 1 min. FAM was used as the fluorescent reporter coupled at the 5′ end of the probe, whereas Block Hole Quencher was conjugated to the 3′ end. Each experiment included three nontemplate controls to detect any template contamination. In addition, a control lacking reverse transcriptase was included for each sample to detect any residual genomic DNA. Standard curves for the quantification of PR and β-actin mRNAs were generated using serial 10-fold dilutions from 10^6 to 10^2 copies of synthesized templates. Q-PCR was performed in triplicate of each sample. The obtained PR mRNA concentration was normalized by the β-actin mRNA value.

Transfections

MCF-7 cells were grown for 48 h in IMEM + 5% FBS till 80% confluence before transfection. Stable transfectants of myr-Akt and KD-Akt, which contains K179M substitution, were gifts from Dr. Adriana Stoica (58). Using Lipofectamine (Invitrogen), a FLAG-PR-B expression vector pSG5-hPR1, which was a generous gift from Dr. K. Horwitz, was cotransfected with the neomycin resistant gene vector pcDNA3.1 (Invitrogen) in a 20:1 ratio (PR vector: neomycin vector). G418 of 600 μg/ml was used to select stable clones that were later verified by Western blot analysis with the PR antibody. All transient transfections were performed using Polyfect reagents (QIAGEN) according to the instruction protocols. For cotransfections with FLAG-PR-B and HA-IRS-1 constructs, 0.1 μg DNA of each plasmid was added to one well in six-well dishes. After 18 h, the culture medium was changed to SFM, and cells were incubated for 12 h before stimulation with 10 nM IGF-I and 10 nM R5020 for 24 h. For transfections with only ERE-tk-luc and PRE-tk-luc constructs, cells in six-well dishes were transfected with 0.2 μg DNA. After 5 h, the serum medium was switched to SFM, and cells were incubated for 6–8 h in the presence or absence of IGF-I. Then the cells were stimulated with 10 nM R5020 10 nM or 10 nM estradiol for 10–12 h. For the study of the inhibitors, cells were preincubated with LY294002 and rapamycin for 30 min before IGF-I treatment in the presence of the inhibitors. To examine the effect of myr-Akt and myr-PI3K on ER and PR activity, cells in six-well dishes were cotransfected with 0.2 μg reporter plasmids and 0.5 μg myr-Akt, myr-PI3K or empty pcDNA3 vector. Transfection time was extended to 16 h to allow sufficient time for exogenous Akt and PI3K expression. Then the serum medium was switched to SFM, and cells were stimulated with estradiol or R5020 for 12 h. Twenty nanograms of a β-galactosidase expression vector pSV-β-Gal (Promega Corp., Madison, WI) were cotransfected as an internal control. Luciferase and β-galactosidase assays were performed using the Promega Corp. assay system.

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Address all correspondence and requests for reprints to: Adrian V. Lee, Ph.D., Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: avlee@breastcenter.tmc.edu.

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Progesterone crosstalks with insulin-like growth factor signaling in breast cancer cells via induction of insulin receptor substrate-2

Xiaojiang Cui[1], ZaWaunyka Lazard[1], Ping Zhang[1], Torsten A Hopp[1] and Adrian V Lee*,[1]

[1]*Breast Center, Baylor College of Medicine, Houston, TX 77030, USA*

Both progesterone and the insulin-like growth factors (IGFs) are critically involved in mammary gland development and also in breast cancer progression. However, how the progesterone and IGF signaling pathways interact with each other to regulate breast cancer cell growth remains unresolved. In this study, we investigated progesterone regulation of IGF signaling components in breast cancer cells. We found that insulin receptor substrate-2 (IRS-2) levels were markedly induced by progesterone and the synthetic progestin R5020 in MCF-7 and other progesterone receptor (PR) positive breast cancer cell lines, whereas IRS-1 and the IGF-I receptor were not induced. The antiprogestin RU486 blocked the R5020 effect on IRS-2 expression. Ectopic expression of either PR-A or PR-B in C4-12 breast cancer cells (estrogen receptor and PR negative) showed that progestin upregulation of IRS-2 was mediated specifically by PR-B. The IRS-2 induction by R5020 occurred via an increase of IRS-2 mRNA levels. Furthermore, progestin treatment prior to IGF-I stimulation resulted in higher tyrosine-phosphorylated IRS-2 levels, increased binding of IRS-2 to Grb-2 and the PI3K regulatory subunit p85, and correspondingly enhanced ERK and Akt activation, as compared with IGF-I-only conditions. Taken together, our data suggest that IRS-2 may play an important role in crosstalk between progesterone and the IGFs in breast cancer cells.
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Keywords: breast cancer; IGFs; IRS-2; phosphorylation; progesterone; R5020

Progesterone is critically involved in the development of the mammary gland and breast cancer, and its effects are mostly mediated by the progesterone receptor (PR) (Graham and Clarke, 1997). The presence of PR in breast tumors is an important predictive indicator of likely responsiveness to endocrine agents (Lapidus *et al.*, 1998). Human PR normally exists in two isoforms, PR-A and PR-B, of 94 and 116 kDa (Kastner *et al.*, 1990), with PR-B containing an additional 164 amino acids at its N terminus. Although the two PR isoforms exhibit similar hormone- and DNA-binding properties, they display different transcriptional activities and are unequally expressed in different tissues and tumors (Wen *et al.*, 1994; Richer *et al.*, 2002).

Progesterone is proliferative in the normal mammary gland (Graham and Clarke, 1997), but its inhibition of breast cancer cell growth in tissue culture has been well documented (Musgrove *et al.*, 1991; Groshong *et al.*, 1997; Lin *et al.*, 1999). Interestingly, progestins have been found to exert a biphasic regulation of breast cancer cell growth – accelerating cells through the first mitotic cell cycle and then arresting them in G1 of the second cycle. At this stage, the cell-cycle progression machinery is poised to restart, as expression of epidermal growth factor and its receptor is increased by progestins (Musgrove *et al.*, 1991; Groshong *et al.*, 1997). Hence, it is proposed that progestins are inherently neither growth proliferative nor growth inhibitory, but rather sensitize breast cancer cells for growth factor and cytokine signals (Lange *et al.*, 1998).

The insulin-like growth factors (IGFs) are key regulators of cell proliferation, survival, and differentiation (LeRoith, 1996). They play an important role in malignant transformation and invasion (Baserga, 1995). Numerous lines of evidence support a role for the IGFs in breast cancer pathogenesis (Lee and Yee, 1995). The IGF-I receptor (IGF-IR), upon activation by the IGFs, phosphorylates the insulin receptor substrates IRS-1 and IRS-2, which are multisite docking proteins that link multiple downstream signaling pathways by binding to a variety of SH2 domain-containing proteins (Yenush and White, 1997). IRSs are also involved in signaling of insulin, interleukins, interferons, and growth hormone, and are implicated in breast cancer growth (Surmacz and Burgaud, 1995; Rocha *et al.*, 1997; Chang *et al.*, 2002). The IRS network of upstream and downstream signaling may place them in a central position to coordinate multiple signaling pathways. IRS-1 and IRS-2, despite their structural and functional similarities, are not completely interchangeable (Bruning *et al.*, 1997).

Recently, much study has been directed to crosstalk between the IGFs and the estrogen receptor (ER) signaling in breast cancer cells (Dupont and Le Roith, 2001), and IRS-1, IRS-2, and IGF-IR have been shown to be induced by estrogen (Lee *et al.*, 1999). However, how the IGFs interact with progesterone in breast cancer is less well defined. In an attempt to elucidate crosstalk and synergism between progesterone and IGF

*Correspondence: Dr AV Lee; E-mail: avlee@breastcenter.tmc.edu
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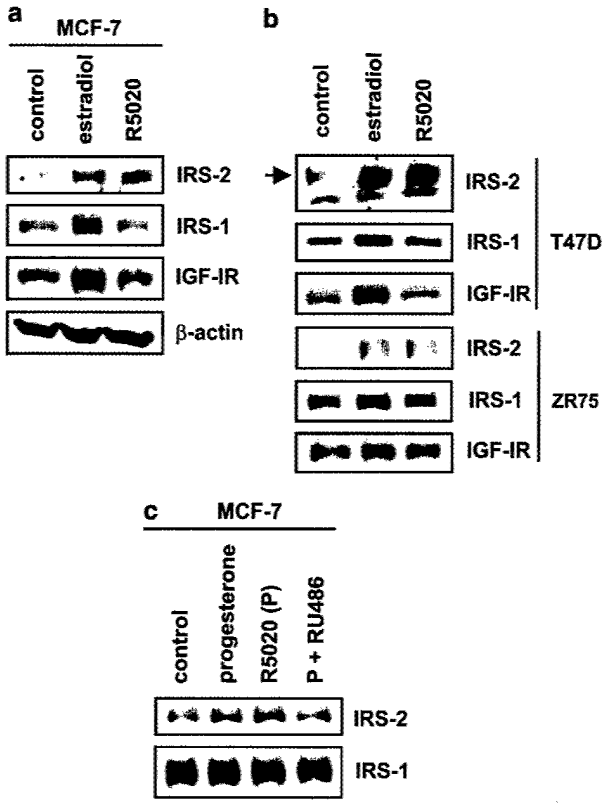

Figure 1 Progesterone induces IRS-2 expression in breast cancer cells. (**a**) MCF-7 cells were grown as described previously (Lee *et al.*, 1999). Cells were first starved for 16 h in serum-free medium (SFM), and then treated for 24 h with 10^{-9} M estradiol or 10^{-8} M R5020. Cell lysate proteins (40 μg) were separated by 8% SDS–PAGE and immunoblotted with antibodies against IRS-1 (Upstate, Lake Placid, NY, USA), IRS-2 (Upstate, Lake Placid, NY, USA), and IGF-IR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β-actin was used as a loading control. (**b**) T47D and ZR75 breast cancer cells were also treated as in (**a**) and subjected to Western blot analysis of IRS-1, IRS-2, and IGF-IR. (**c**) MCF-7 cells were treated for 24 h with 10^{-8} M progesterone, 10^{-8} M R5020, or 10^{-8} M R5020 plus 10^{-6} M RU486. Cell lysates were subjected to Western blot analysis of IRS-1 and IRS-2

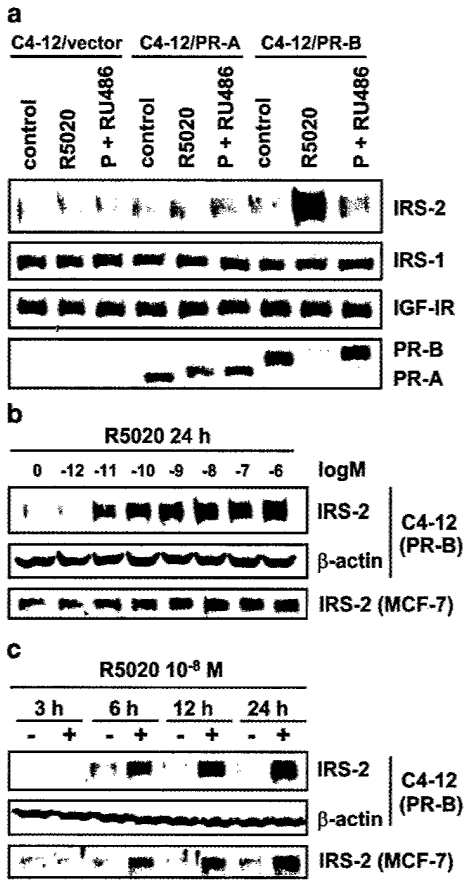

Figure 2 PR-B mediates the progesterone upregulation of IRS-2 in breast cancer cells. PR-A or PR-B cDNA was stably transfected into a specially selected MCF-7 cell sub-line C4-12 which is ER−/PR−. (**a**) Vector-, PR-A-, and PR-B-transfected C4-12 cells were stimulated for 24 h with 10^{-8} M R5020, 10^{-6} M RU486, or both together. Cell lysate proteins (40 μg) were separated by 8% SDS–PAGE and immunoblotted with antibodies against IRS-1, IRS-2, IGF-IR, and PR (DAKO, Carpinteria, CA, USA). (**b**) C4-12/PR-B and MCF-7 cells were stimulated for 24 h with increasing concentrations of R5020 in a dose response experiment. Cell lysate proteins were separated by 8% SDS–PAGE and immunoblotted with IRS-2 or β-actin antibodies. (**c**) C4-12/PR-B and MCF-7 cells were stimulated with 10^{-8} M R5020 or vehicle for increasing periods of time in the time course assay. Cell lysates were immunoblotted with IRS-2 or β-actin antibodies

signaling in breast cancer cells, we have now investigated progesterone regulation of the IGF signaling pathway.

As a first step, we treated ER and PR positive (ER+/PR+) MCF-7 cells in serum-free medium with the synthetic progestin R5020, and then tested how expression of IRS-1, IRS-2, and IGF-IR was affected. Immunoblot analysis of total cell lysates demonstrated that a single dose of 10^{-8} M R5020 for 24 h remarkably increased IRS-2 levels in MCF-7 cells, while it did not alter the expression of IRS-1 and IGF-IR (Figure 1a). This is in contrast to the effect of estradiol, which upregulates all these proteins. The R5020 effect on IRS-2 was also observed in other ER+/PR+ breast cancer cell lines like T47D and ZR75 (Figure 1b). Similar but slightly weaker induction of IRS-2 also occurred with a single dose of 10^{-8} M progesterone (Figure 1c), probably due to progesterone's much shorter half-life in cell culture than R5020 (Groshong *et al.*, 1997). Moreover, the antiprogestin RU486 blocked the IRS-2 upregulation by R5020 in MCF-7 cells (Figure 1c), suggesting that, as expected, the IRS-2 induction by progestins is mediated by PR in breast cancer cells.

To better define the role of progesterone and the two PR isoforms on IRS-2 in breast cancer cells, we established the expression of either PR isoform by stably transfecting PR-A or PR-B cDNA into C4-12 cells, a specifically selected MCF-7 cell subline that does not have detectable ER or PR (Oesterreich *et al.*, 2001). In PR-B-transfected C4-12 cells, IRS-2, but not IRS-1 or IGF-IR, was upregulated significantly by R5020 (Figure 2a), while PR-B by itself had no ligand-independent induction of IRS-2 expression. In addition, the antiprogestin RU486 completely blocked the IRS-2 upregulation by R5020 in the C4-12/PR-B cells. As expected, the R5020 effect on IRS-2 was not observed in parental or vector-transfected C4-12 cells. The R5020-

mediated effects were similar in several different C4-12/PR-B clones. In contrast, ectopic expression of PR-A in C4-12 cells did *not* render IRS-2 progestin-inducible (Figure 2a), which re-emphasizes the fact that the two PR isoforms possess distinct gene transcriptional activities and that PR-B is transcriptionally more active (Richer *et al.*, 2002). Thus, our data suggest that it is PR-B that mediates the progestin effect on IRS-2 upregulation. The induction of IRS-2 by R5020 over the untreated control was noticeably stronger in C4-12/PR-B cells than in MCF-7 cells (see Figure 1), perhaps due to the finding that C4-12/PR-B cells have 3–5-fold higher PR-B protein levels than MCF-7 cells. Another explanation may be that the PR-A also present in MCF-7 cells might act as a repressor of PR-B in regulating IRS-2 expression (Vegeto *et al.*, 1993).

To extend the study of progesterone regulation of IRS-2 in C4-12/PR-B cells, we performed a dose response assay using a 24 h stimulation. As shown in Figure 2b, R5020 at concentrations as low as 10^{-11} M dramatically increased IRS-2 levels. Maximal induction of IRS-2 expression occurred at 10^{-9} M R5020 and higher. In a time course experiment using 10^{-8} M R5020, we found that elevated IRS-2 protein levels were visible after 6 h of R5020 treatment and continued to increase throughout the 48 h time period (Figure 2c). Similar dose response and time course results were also observed in MCF-7 cells (Figure 2b and c). Taken together, these data suggest that the induction of IRS-2 is very sensitive to progesterone in PR-B-expressing breast cancer cells.

To assess whether progesterone uses transcriptional mechanisms to modulate IRS-2 levels, we first examined changes of IRS-2 mRNA concentrations in MCF-7 cells treated with R5020. RT–PCR analysis demonstrated that 10^{-8} M R5020 treatment for 3 h significantly increased IRS-2 mRNA levels, while RU486 impaired this IRS-2 mRNA induction by R5020 (Figure 3a). In the earlier time course experiment, IRS-2 protein was barely upregulated by R5020 by the 3 h time point, suggesting that the elevation of IRS-2 mRNA levels occurs prior to that of IRS-2 protein. To confirm that the progestin upregulation of IRS-2 was via transcriptional mechanisms, we preincubated MCF-7 cells with the transcription inhibitor 5,6-dichlorobenzimidazole riboside (DRB), and then stimulated the cells with R5020 in the continual presence of DRB. Not surprisingly, the IRS-2 mRNA upregulation by R5020 was suppressed by the DRB pretreatment (Figure 3a). The result was also confirmed by RNA protection assay (data not shown). In line with this, immunoblotting showed that the IRS-2 protein increase by R5020 was also blocked by the DRB pretreatment (Figure 3b), confirming that induction of IRS-2 mRNA synthesis is a prerequisite for the IRS-2 protein increase by progestins.

Furthermore, when hemagglutinin (HA) epitope-tagged IRS-2 cDNA driven by the CMV promoter was either transiently or stably transfected into MCF-7 and other breast cancer cell lines, R5020 could not upregulate HA-IRS-2 levels in the transfected cells (data not shown), suggesting that the progesterone impact on IRS-2 expression in breast cancer cells does not occur via alteration of protein translation or stability, but rather relies on the original IRS-2 promoter context.

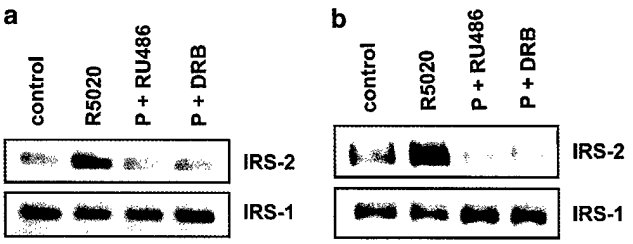

Figure 3 Progesterone induces IRS-2 mRNA levels. (**a**) MCF-7 cells were stimulated for 3 h with 10^{-8} M R5020, R5020 plus 10^{-6} M RU486, or R5020 in the presence of the transcription inhibitor DRB (50 μM) after preincubation with DRB for 30 min. Total RNA was then isolated and 100 ng RNA was used in RT–PCR of IRS-1 and IRS-2, which was conducted using Superscript II reverse transcriptase (Invitrogen) and gene-specific primers (IRS-1: 5′-GCTGCTAGCATTTGCAGGCCTAC-3′, 5′-CTGACGGTCCTC TGGCTGCT-3′; IRS-2: 5′-TTGACGTCGGGCGTGAAGAG GCT-3′, 5′-CTCTTTCACGATGGTGGCCTCC-3′). The PCR was performed at an annealing temperature of 60°C and was subjected to 30 cycles. Products were revealed by ethidium bromide staining under UV after agarose gel electrophoresis. (**b**) MCF-7 cells were stimulated for 24 h with 10^{-8} M R5020, R5020 plus 10^{-6} M RU486, or R5020 in the presence of DRB after preincubation with DRB for 30 min. Cell lysate proteins were separated by 8% SDS–PAGE and immunoblotted with IRS-2 or IRS-1 antibodies

In summary, these data suggest that the progesterone effect on IRS-2 in breast cancer cells is mediated by transcriptional mechanisms. Surprisingly, IRS-2 was not among the 94 progesterone-regulated genes identified in a recent study using T47D breast cancer cells and cDNA microarrays (Richer *et al.*, 2002). One explanation for the apparent discrepancy between this and our studies might be that IRS-2 mRNA is in low abundance in T47D cells and thus may not have been detected in the microarray analysis.

Since the IGFs utilize IRSs to transduce their signals in cells, we next examined how progestin's alteration of the IRS-2 levels might sensitize breast cancer cells to IGF signals. We preincubated MCF-7 cells with 10^{-8} M R5020 for 24 h and then stimulated the cells with 100 ng/ml IGF-I for 10 min. Immunoprecipitation with IRS-2 antibodies followed by immunoblotting demonstrated that R5020 pretreatment increased IRS-2 levels and consequently, the levels of tyrosine-phosphorylated IRS-2 induced by IGF-I. IRS-2 mobility was also shifted upward due to phosphorylation (Figure 4a). R5020 by itself could not activate IRS-2. An important feature of the IRS-mediated response to IGF-I is docking of Grb-2 and the PI3K regulatory subunit p85, which leads to activation of the Ras/ERK and PI3K/Akt signaling pathways. As shown in Figure 4a, R5020 enhanced the IGF-I-triggered association of p85 and Grb-2 with tyrosine-phosphorylated IRS-2, most probably due to upregulation of activated IRS-2 levels, since total cellular p85 and Grb-2 levels were not changed by R5020 (Figure 4a). In contrast, the association of p85 and Grb-2 to IRS-1 (not induced by progestins) after IGF-I stimulation was not enhanced by R5020. Moreover, upregulation of IRS-2 did not

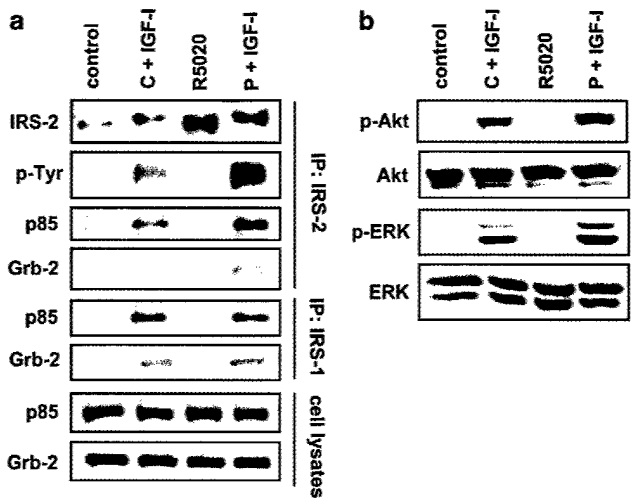

Figure 4 Progesterone enhances IGF-I signaling mediated by IRS-2. (**a**) MCF-7 cells were first treated for 24 h with vehicle or 10^{-8} M R5020, and then stimulated with 100 ng/ml IGF-I for 10 min. Cell lysates (500 μg) were immunoprecipitated with IRS-2 antibodies at a 1:100 dilution. Aliquots of the immunoprecipitates were subjected to immunoblot analysis with the p85 (Upstate) and Grb-2 (Upstate) antibodies. Tyrosine phosphorylation of IRS-2 was detected with the specific phosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY, USA). As a comparison, the association of p85 and Grb-2 with IRS-1 was also examined using immunoprecipitation with IRS-1 antibodies. (**b**) MCF-7 cells were first treated for 24 h with vehicle or 10^{-8} M R5020, and then stimulated with 100 ng/ml IGF-I for 10 min. Cell lysates were separated by 8% SDS–PAGE and immunoblotted with antibodies against total Akt and ERK (Cell Signaling, Beverly, MA, USA) and their corresponding phosphorylation-specific antibodies (Akt: Ser 473; ERK: Thr202/Tyr204)

impede the association of p85 and Grb-2 to IRS-1 upon IGF-I stimulation.

Since elevated IRS-2 levels were associated with increased p85 and Grb-2 binding, we measured subsequent Akt and ERK activation, using their phospho-specific antibodies. Immunoblot analysis revealed that activation of ERK and Akt triggered by IGF-I was higher after R5020 pretreatment, even though levels of total ERK and Akt were unaffected (Figure 4b). Nor did R5020 by itself cause detectable phosphorylation of ERK and Akt. We consistently obtained similar results, that is, R5020 treatment led to 50–100% increase of IGF-I-stimulated phospho-Akt and phospho-ERK. It may be that we did not observe a more dramatic increase of Akt and ERK activation under the R5020 condition because the basal amount of IRS-1 is much higher than that of IRS-2, and IRS-1 is the predominant signaling molecule activated by IGF-I in MCF-7 as well as other ER+ breast cancer cells (Jackson *et al.*, 1998). Despite a considerable increase in expression after R5020 treatment, IRS-2 is still unable to fully overcome the dominant role of IRS-1 in IGF signal transduction.

It is also noted that estradiol seems to have a more prominent effect than progestins on enhancing IGF-I-induced activation of ERK in MCF-7 cells (Lee *et al.*, 1999; Dupont and Le Roith, 2001), which is probably due to estrogen's induction of IRS-1 and IGF-IR, as well as IRS-2, whereas progesterone only induces IRS-2. Taken together, these data suggest that R5020 potentiates IGF-I signaling in breast cancer cells through upregulation of IRS-2. Given that IRS-2 has been shown to interact with and be activated by other pathways such as integrins (Shaw, 2001), it is possible that progesterone also sensitizes breast cancer cells to IRS-2-mediated integrin signaling.

Previously, IRS-2 was identified as a progesterone response gene in PR-transfected HeLa cells, using differential display (Vassen *et al.*, 1999). So far, only a few PR-regulated genes have been characterized in breast cancer. This study represents the first attempt to characterize progesterone regulation of IRS signaling in breast cancer cells. Since IRSs are involved in effects of the IGFs, insulin, growth hormones, interleukins, and interferons, increased IRS-2 in cells may contribute to the promotion of cell proliferation, survival, and motility by these mitogens. Recently, the Women's Health Initiative, after a study of more than 16 000 women, concluded definitively that combined estrogen and progestin hormone therapy increases the risk of invasive breast cancer by 25% as compared to women taking placebo (Rossouw *et al.*, 2002). In another study (Schairer *et al.*, 2000), it was found that the risk of developing breast cancer was higher in women on estrogen and progestin therapy than in women who used estrogen therapy alone, which was confirmed by Ross *et al.* (2000). These findings raised the question of why the addition of progestin to hormone replacement therapy would markedly enhance the risk of breast cancer relative to estrogen use alone. This enigma might be partly explained by the notion that progestins prime cells for extracellular signals through upregulation of pivotal cell signaling components. Our data presented here imply that progesterone may sensitize cells to signaling pathways that involve IRS-2 function. Future studies will be needed to determine the importance of the progesterone regulation of IRS-2 in breast cancer initiation and progression as well as normal mammary epithelial cell growth.

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